REVIEW ARTICLE

Tuberculosis into the next century

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Introduction

Tuberculosis is an ancient disease, the presence of which has been inferred from as early as 4000 BC from Egyptian tomb paintings and by examination of mummies for spinal tuberculosis [1]. Tuberculosis may have emerged during neolithic times when human populations increased and aggregated and cattle were domesticated [2]. Examination of a skeleton dating from the fourth millennium BC, excavated from Areneda Cave in Liguria, Italy, demonstrated evidence of spinal tuberculosis [3]. More precise evidence will be obtained by application of polymerase chain reaction technology to DNA extracted from infected sites in ancient corpses. Tuberculosis was present in America prior to the arrival of Columbus [4, 5]. The earliest evidence of tuberculosis in Britain comes from Roman times [6]. However, tuberculosis was certainly well-described by the time of Hippocrates and Aristotle in the fifth century BC when it was described as phthisis, which was translated into English as consumption [7]. Fracastero (1483–1553) deduced that phthisis was infective and Sylvius (1614–1672) recognised the characteristic nodules at autopsy which he named tubercles [8]. In 1868 Villemin, a French military surgeon, published a treatise on the epidemiology of tuberculosis and succeeded in transmitting the disease to animals [9]. In 1882 Robert Koch reported the isolation of the tubercle bacillus to the Berlin Phthisiological Society [10, 11]. This together with the discovery of staining techniques by Ehrlich (1885) and of X-rays by Roentgen (1895) provided powerful tools to conquer the disease.

Tuberculosis has exerted a great influence on man's thinking. Bunyan described it as 'the captain of all the men of death', and it has been depicted as a great killer in music (*La Bohème*) and literature (*Mountain*, Thomas Mann). Indeed, in the 19th Century

it was feared that tuberculosis might bring about the end of European civilisation.

It is thought that in 1650 tuberculosis accounted for almost 20% of all deaths in urbanised areas of England and Wales but this had declined to 13% by 1715 [12]. Thereafter the incidence increased to a peak in the first half of the 19th century. During this time most cities in the eastern USA had case rates of tuberculosis of > 400/100 000 population and 15–20% of deaths were attributable to tuberculosis [8]. A similar pattern was obtained for western Europe but was delayed for several decades in eastern Europe. In England and Wales the death rate from tuberculosis from 1851 to 1860 was 3478/10⁶ population [13]. By 1901–1910 it had halved (1646/10⁶) and by 1951 had dropped to one-tenth (310/10⁶). The most profound drop was in Denmark where in 1885 the death rate was 3000/10⁶ population but this had dropped to 50/10⁶ by 1955 [13]. The exceptions to this inexorable decrease in tuberculosis were in those countries most affected by the two World Wars. For example, in the Netherlands the rate rose by 208% in Amsterdam and by 111% in the rest of the country in 1945 [13] but it has now decreased to the lowest rate in Europe. Although there has been a remarkable decline in tuberculosis in developed countries, in sub-Saharan Africa and parts of Asia it is still a major problem. The WHO estimates that c. 3 million people die each year from tuberculosis and that 20% of deaths occurring in those aged 15–59 years are due to tuberculosis.

The decline of tuberculosis in developed countries can be related to improvements in social and hygienic conditions and nutrition, to improved chemotherapy and to immunisation. Indeed, we have become complacent about the danger of tuberculosis. This complacency has been somewhat disturbed by recent happenings. Firstly, the emergence of HIV and AIDS has increased the susceptibility of a small proportion of the population to tuberculosis and provided a new reservoir of the disease. Secondly, multi-drug-resistant

strains of *Mycobacterium tuberculosis* have emerged, rendering current chemotherapeutic regimens ineffective [14]. Finally, there has been an unexpected increase in tuberculosis in developed countries [15]. For example, in England and Wales from 1988 to 1993, 8000 more cases of tuberculosis were notified than predicted from previous trends [16]. This increase was not entirely explicable by the emergence of AIDS, but is closely related to poor socio-economic conditions [17]. Although immigrant populations have been long recognised to be at high risk of developing tuberculosis [18], deteriorating social conditions appear to have a stronger link than migration in the recent increase in UK tuberculosis notification [19, 20].

The second in a series of infectious disease symposia held at the Liverpool School of Tropical Medicine addressed the problem of tuberculosis. A multidisciplinary team of speakers was asked to cover wide ranging topics that included the molecular biology of drug resistance, BCG and new immunisation strategies, molecular diagnosis and epidemiology, new therapeutic agents, mycobacterial taxonomy, and the clinical expression of tuberculosis in children, adults and patients with AIDS.

MOLECULAR DIAGNOSIS AND EPIDEMIOLOGY OF TUBERCULOSIS

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Introduction

The explosive development in the last 10 years of the technology of molecular biology has provided a host of potential applications for diagnostic microbiology. Initially, nucleic acid probes were applied to both cultured bacteria and clinical specimens. Subsequently, various radioactive [21] and non-radioactive [22] probes have been found to be most useful for the rapid identification of cultured mycobacteria. Nucleic acid amplification techniques, such as PCR, can be applied to the detection of specific and non-specific mycobacterial gene sequences in clinical specimens, cultures and environmental samples to provide an extremely sensitive and rapid diagnostic test. PCR may also be used to produce probes for the confirmation of amplimer identity [23], RFLP typing [24] to amplify species-specific rRNA sequences for species identification [25] and direct PCR typing [26].

Molecular techniques for the diagnosis of mycobacterial infection

The nucleic acid amplification-based methods for the

detection and identification of mycobacteria fall into one of three types: (i) amplification of genus-specific sequences; (ii) amplification of genus-specific sequences followed by oligonucleotide probing to give a species-specific test; (iii) amplification of species-specific sequences. A very wide range of target sequences has been used, although in the case of genus-specific primer pairs, the genes encoding the 65-kDa antigen are popular [27, 28] and for species-specific primers those based on IS6110/IS986 [e.g., refs 29, 30]. The first PCR test for the diagnosis of tuberculosis was based on the 65-kDa antigen sequence and used two oligonucleotide probes to hybridise with the amplimer, which was visualised on an agarose gel [27]. Six sputa, three lymph nodes and a specimen of pus, which were all positive for *M. tuberculosis* by microscopy or culture, or both, gave positive results by PCR. A further two gastric aspirates, from a patient receiving anti-tuberculosis therapy, were negative by conventional methods but were positive by PCR; all the other negative specimens were also negative by PCR. The great potential of PCR in diagnosing tuberculosis was revealed, particularly in relation to speed and sensitivity. The first primers to amplify a species-specific sequence were those described by Shankar *et al.* [31] who used the sequence encoding the MPB64 antigen that had been used previously in ELISA tests. Similar results to the first study by Brisson-Noel *et al.* [27] were obtained. Subsequently a very large number of primer pairs to a wide range of targets were described by different groups. Space does not permit a comprehensive review of those papers but they are described elsewhere [23, 32]. Comparison of performance of different PCR protocols is difficult due to the comparative paucity of clinical specimens and lack of a highly sensitive 'gold standard' method. Because of the presence of multiple copies of IS6110 in most strains of *M. tuberculosis*, there are more methods based on this target than others, as there is also an element of 'natural' amplification. In two studies of sputum, lavage and pleural fluid specimens, the primers for the 65-kDa antigen gene sequence were compared with a pair for the IS6110 gene sequence [33, 34]. The IS6110 primers were generally more sensitive and specific. A number of patients with inactive tuberculosis and contacts had specimens that were PCR positive, presumably because of the presence of very small numbers of live or dead bacteria that may be present in macrophages.

It has become clear that the method used to extract DNA from specimens has a great influence on the sensitivity and specificity of PCR protocols. Many methods are used; they usually include boiling for 10–30 min in buffers containing various alkaline detergents [23, 27]. However, good results can be obtained with simple methods, such as boiling, mechanical disruption [35] and sonication [36]. The last study found that sonication detected 10–10² cfu whereas the other techniques (proteinase K/detergent; boiling in detergent; freeze/thaw) detected 10³ cfu. Whilst all manip-

ulations with *M. tuberculosis* require comprehensive safety protocols, the extraction step can be particularly prone to cross-contamination of the operator or environment. It has been found that specimens must be held for a minimum period of 5 min at 100°C to ensure complete killing [37]. Commercially available chelating agents, such as Chelex 100® (BioRad) have also been used successfully to extract DNA from *M. tuberculosis* [38].

False negative results in specimens may be obtained due to the presence of inhibitors of the PCR reaction, such as haemoglobin and proteins [23, 39]. Initially, some workers spiked the sample with small amounts of DNA and primers not related to mycobacteria such as the cloning vector pUC19 [40]. The disadvantage is that the specific mycobacterial primers are not being controlled. This is avoided by the methods of Eisenach and colleagues [41], in which the recognition sites for the IS6110-directed primers were cloned into pUC19 together with a stuffer fragment of 600 bp from *Salmonella typhimurium*. The vector DNA can then be used to control the IS6110 primers and yields a larger product than that arising from the IS6110 sequences in the specimens. This approach was taken further by Kolk *et al.* [39] by inserting the modified IS6110 recognition sequence into *M. smegmatis*, thus allowing a small number of these cells to be added to each specimen, resulting in control, not only of primers, but also of DNA loss during processing. Contamination is always a potential problem with PCR, but more so when using a slow-growing group of bacteria widely distributed in the environment. Cross-contamination of negative specimens with *M. tuberculosis* is well recognised as causing problems in conventional bacteriological procedures and will lead to a positive PCR result. Contamination of reagents and specimens with amplicon (amplified PCR product) is the most commonly recognised problem and has been reported on a number of occasions [23]. Physical separation of amplicon, set-up areas and equipment is sensible; decontamination of surfaces with sodium hypochlorite in addition to the substitution of dUTP for dTTP and treatment with uracil-N-glycosylase has been described for the removal of amplicon contamination [42].

The problems of contamination were emphasised by a blinded study of seven laboratories experienced in PCR for *M. tuberculosis*. Laboratories reported false positive rates ranging from 3–20% up to 77% [43]. Only two of the seven laboratories correctly identified all of the positive simulated specimens of sputum containing 10⁴ cfu. This perturbing finding demonstrates that current protocols even in apparently expert laboratories are far from perfect. The authors concluded that effective monitoring of specimen processing, in addition to that required to check sensitivity and specificity of PCR, is essential for reliable diagnosis of tuberculosis.

Non-PCR-based amplification techniques

Several non-PCR-based amplification techniques have been applied to the diagnosis of mycobacterial infections. Some that have been reported are: strand displacement assay (SDA) developed by Becton Dickinson, transcription-mediated amplification (TMA) developed by Gen-Probe and reporter phage systems [44]. At the time of writing in the UK, only the TMA is available commercially and has been evaluated by a number of groups [45–47]. The principle of the system is that specific rRNA is amplified isothermally by an autocatalytic process, the products being detected with an acridium-labelled DNA probe in a homogeneous solution hybridisation following a reverse-transcriptase step. The test has been formatted for high throughput clinical laboratories and appears to perform well in evaluations; however, it is expensive.

Molecular epidemiology

The identification of subtypes of *M. tuberculosis* has been a long-standing problem that has dogged epidemiologists and microbiologists [48]. The development of molecular fingerprinting techniques that identify subtypes that are related to the apparent epidemiology of cases promises to be one of the most exciting developments in the study of tuberculosis. Most typing systems, including the one that has been accepted as the standard technique, are based on the insertion sequence IS6110 [29]. Insertion elements are sequences of DNA bounded by short inverted repeated DNA sequences together with a direct repeat of the target sequence (*c.* 3 bp) which are capable of movement within a replicon by a process analogous to transposition. Several different elements have been described and used in molecular typing (Table 1) [49–55]. The insertion sequence IS6110 is an element usually present in 1–25 copies (although occasional strains lack a copy) in *M. tuberculosis* [49, 56]. In the standardised technique [24], genomic DNA is cut with the restriction endonuclease *Pvu*II and the fragments are separated on an agarose gel and transferred to a nylon membrane. The restriction endonuclease *Pvu*II has one asymmetric site within IS6110 and the genomic DNA is probed with IS6110 DNA generated from DNA to the right hand side of this site. The resulting fingerprint will contain a number of bands which correspond to the number of copies of IS6110 in an individual strain. This method is described in detail elsewhere [57]. This technique has been found to produce a level of typability and strain discrimination that is appropriate for the investigation of cross-infection by *M. tuberculosis*. Because insertion elements are mobile, it has been suggested that the evolution of the RFLP (restriction fragment length polymorphism) occurs in individual patients and leads to an over-discriminatory typing result [58]. However, provided that it is accepted that differences in patterns of only one or at most two bands are found in clonal isolates, epidemiologically reliable

Table 1. Insertion sequences and repetitive DNA of *M. tuberculosis* that have been used for typing (after Small and van Embden [48])

Element	Size (bp)	Source	Host range	Copy no.	References
IS6110	1355	<i>M. tuberculosis</i>	<i>M. tuberculosis</i> complex	0–25	29, 49, 50
IS1081	1324	<i>M. bovis</i>	<i>M. tuberculosis</i> complex	5–7	51, 52
			<i>M. xenopi</i>		51, 52
DR	36	BCG	<i>M. tuberculosis</i>	10–50	53, 50
MPTR	10	<i>M. tuberculosis</i>	<i>M. tuberculosis</i> complex	>100	53, 54
			<i>M. gordonae</i>		
			<i>M. kansasii</i>		
			<i>M. asiaticum</i>		
			<i>M. gastri</i>		
			<i>M. szulgai</i>		
PGRS	30	<i>M. tuberculosis</i>	<i>M. tuberculosis</i> complex	>100	26
GTG	15	Synthetic	Unknown	>5	55

typing can be achieved. In studies designed to look at this issue, the authors concluded that RFLPs examined with *Pvu*II digestion and IS6110 probing are very stable [59, 60].

There are many examples in the published literature where RFLP typing has solved difficult epidemiological problems. Cross-infection has been clearly demonstrated amongst HIV-infected individuals [61, 62], in renal units [63] and in persons emigrating from one country to another [64, 65]. In one particularly striking study [66], analysis of a large proportion of the isolates of *M. tuberculosis* (61%) from culture positive cases of tuberculosis in Berne, Switzerland, showed that one particular strain type was highly prevalent in homeless drug-users and that unsuspected infection in the general population frequenting a particular restaurant was occurring. There are important social implications in that the greater use of such typing methods can be used to measure the mobility of infectious people and to monitor the effectiveness of directly observed therapy. IS6110 RFLP typing has also been applied in areas of the world where tuberculosis is endemic, such as equatorial Africa [67] and Tunisia [68]. The Tunisian study was able to show the persistence of unrecognised micro-epidemics which can prevent effective control. Within the laboratory, the technique has been used successfully to demonstrate cross-contamination of laboratory cultures [69]. The utility of techniques for typing most strains of the *M. tuberculosis* complex was demonstrated in a comparative study of various repetitive elements [56]. Some strains of *M. tuberculosis* have only a single copy of IS6110 which greatly limits strain differentiation [56]. Such strains have been distinguished by hybridisation of genomic DNA with probes to a polymorphic GC-rich repetitive sequence (PGRS), (GTG)⁵ or a 36-bp DR (direct repeat) sequence [53, 55, 56]. *M. bovis* typically carries a single copy of IS6110, but isolates from cattle have been differentiated successfully by probing for the repetitive element (PGRS) and the insertion sequence IS1081 [70, 71].

Unfortunately, IS6110 RFLP analysis requires the

preliminary growth of cultures to produce µg quantities of pure unsheared DNA, followed by a lengthy blotting and hybridisation protocol. Therefore, there has been considerable interest in developing PCR-based typing systems. In one approach, primers amplifying away from each other, but located in IS6110, produced polymorphic banding patterns which DNA sequencing confirmed as arising from priming between adjacent copies of IS6110 and non-specific binding sites [26]. Arbitrary primed PCR has also been described [72, 73]. In both cases, the technique was apparently useful in distinguishing clusters of isolates, but applicability to a wider range of isolates is unclear. The primers used in both cases were derived from IS6110 sequences. Insertion sequence amplotyping with primers based on the major polymorphic tandem repeat (MPTR) sequence and nested primers in IS6110 have been reported to be capable of typing not only cultured isolates, but also directly from sputum specimens [74]. The technically complex method of mixed-linker PCR has been used [75] which has the advantage of generating the same pattern as IS6110 RFLP analysis. A simpler, but similar technique is ligation-mediated PCR, although this does not generate the same pattern [76]. The variable sequence upstream of the *katG* gene has also been used to subdivide strains, but the level of discrimination was poor [77]. However, the principle of amplifying a junction sequence between two genes (in this case two copies of IS6110) has been exploited to identify rapidly isolates of the multi-drug-resistant strain W of *M. tuberculosis* found in New York [78].

Conclusions

Molecular techniques have had an enormous impact on the study and control of disease caused by *M. tuberculosis*, mainly via research groups. We are now entering a phase of consolidation in which the discoveries of those groups need to be developed and evaluated for their applicability to routine diagnostic microbiology. However, it is already evident that major developments are taking place in the areas of specimen examination, identification of cultured mycobacteria and strain differentiation. In particular, the availability of

molecular typing methods could have profound implications for the management and control of tuberculosis in the world, assuming that the underlying social conditions associated with the current epidemic of tuberculosis are not alleviated.

MOLECULAR BASIS OF DRUG RESISTANCE IN *M. TUBERCULOSIS*

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Introduction

Molecular mechanisms of drug resistance in *M. tuberculosis* remained largely unknown until recently. The recent resurgence of tuberculosis [79], along with outbreaks of multi-drug-resistant tuberculosis (MDR-TB) [80], has stimulated a great deal of interest in understanding the molecular basis of drug resistance in *M. tuberculosis*. Tremendous progress has been made recently in this area thanks to the application of mycobacterial molecular genetics [35, 81–84]. In contrast to drug resistance in many other bacterial pathogens, plasmids or transposons have not been found to cause drug resistance in *M. tuberculosis*; rather, chromosomal mutations are responsible. Recent molecular analysis of some MDR-TB strains suggests that the MDR phenotype is caused by sequential accumulation of individual mutations in separate genes, not by novel mechanisms due to a single mutagenic event [85]. Broadly speaking, based on the specificity of anti-TB drugs, drug resistance mechanisms in *M. tuberculosis* can be divided into specific and non-specific mechanisms. The tuberculosis-specific drugs (isoniazid, pyrazinamide, ethambutol etc.) attack unique metabolic pathways in *M. tuberculosis* and are relatively ineffective against other bacteria. Mechanisms of resistance to these drugs are unique to *M. tuberculosis* and so far only isoniazid (INH) resistance mechanisms have been characterised [81, 82]. The mechanisms of resistance to pyrazinamide and ethambutol are yet to be identified. However, the mechanisms of resistance to non-tuberculosis-specific drugs (streptomycin, rifampicin, fluoroquinolones) which are broad spectrum antibiotics have been well advanced. Their mechanisms of resistance in *M. tuberculosis* are analogous to those

found in other bacteria (see Table 2) and will not be discussed in detail here (for review, see refs 86 and 87). The focus of this presentation is on the molecular mechanisms of INH resistance in *M. tuberculosis*.

Mechanisms of INH resistance

Isoniazid (INH) is a key component of anti-tuberculosis chemotherapy and its use in combination with other anti-tuberculosis drugs has had a great impact on the control of tuberculosis. An interesting phenomenon, first observed by Middlebrook in the early 1950s, is that clinical isolates of *M. tuberculosis* resistant to INH often lose catalase and peroxidase activity, and at the same time also show reduced virulence in a guinea-pig model [88]. Furthermore, among INH-resistant *M. tuberculosis* strains (MIC 0.2–100 mg/L), there is an inverse correlation between the level of INH resistance and the loss of catalase and peroxidase activity, i.e., the more resistant a strain is to INH, the less enzyme activity it has [89]. Investigators have long wondered what roles catalase and peroxidase play in the INH resistance and susceptibility of *M. tuberculosis*.

Recently, we cloned a single catalase gene, *katG*, encoding a bifunctional enzyme with both catalase and peroxidase activities from *M. tuberculosis* [81]. With this cloned *katG* gene as a probe, a *katG* deletion was identified in some highly INH-resistant clinical isolates of *M. tuberculosis* [81]. To determine whether the loss of catalase activity (either due to *katG* gene deletion or point mutations) is the cause of INH resistance, INH-resistant *M. tuberculosis* strains that were defective in catalase activity were transformed with a functional *katG* gene. Transformation with the *katG* gene resulted in complete restoration of INH sensitivity to these strains resistant to different concentrations of INH [90]. This suggested that loss of the catalase- peroxidase enzyme is the cause rather than just an accompanying phenomenon in INH resistance. That deletion of a gene is the cause for drug resistance is surprising, since many drug resistances are caused either by acquisition of a plasmid or transposon, or point mutations in the chromosomal genes. For example, kanamycin resistance in Enterobacteriaceae can be transferred by plasmids containing the gene encoding an aminoglycoside-modifying enzyme [91].

The current working model for understanding INH resistance due to *katG* mutations [86] is that catalase-

Table 2. Mechanisms of drug resistance in *M. tuberculosis*

Drug	Specificity for <i>M. tuberculosis</i>	Mutations responsible for drug resistance		References
Isoniazid	Specific	<i>katG</i>	<i>inhA</i>	81, 82
Rifampicin	Non-specific	<i>rpoB</i>		35
Streptomycin	Non-specific	<i>rpsL</i> (S12)	<i>rm</i> (16SrRNA)	83
Fluoroquinolone	Non-specific	<i>gyrA</i>		84

peroxidase of *M. tuberculosis* is involved in the activation of INH, generating reactive radicals possibly attacking multiple targets in *M. tuberculosis* [92, 93]. Loss of catalase-peroxidase activity, either due to *katG* deletions or point mutations, causes INH resistance. While the details of INH activation by the catalase-peroxidase enzyme are yet to be worked out, it is certain that the enzyme is needed for susceptibility of *M. tuberculosis* to INH based on *katG* transformation studies [90]. One of the targets known to be affected by INH is the mycolic acid synthesis pathway [89]. However, the detailed biochemical mechanism for such inhibition by INH is not clear. The recent identification of InhA protein from *M. tuberculosis* with homology to an enzyme involved in fatty acid biosynthesis in *Escherichia coli* should provide a step towards understanding the INH inhibition of mycolic acid synthesis in mycobacteria [82].

Among INH-resistant *M. tuberculosis* strains, *katG* deletion seems to be a relatively rare event and the percentage of such *katG* deletion strains may vary and has not yet been systematically analysed. From a limited number of INH-resistant strains (*c.* 50) examined, *katG* deletion appeared to account for *c.* 5–10% of INH-resistant *M. tuberculosis* strains. Of the remaining strains, most have point mutations in the *katG* gene [85], and a few have point mutations in a second gene, *inhA*, which has also been implicated in INH resistance [82]. The *inhA* gene from *M. tuberculosis* and *M. smegmatis* (a fast-growing soil mycobacterium) encodes a 32-kDa protein with homology to an *E. coli* enzyme (EnvM) involved in fatty acid biosynthesis. Point mutations in the *inhA* gene (Ser to Ala change at amino-acid residue 94) was found to confer a low level of INH resistance as well as ethionamide (a structural analogue of INH) resistance in *M. smegmatis* by allelic exchange experiments (replacing the wild-type gene with the mutant *inhA*). While the allelic exchange experiment remains to be done for *M. tuberculosis*, the same Ser to Ala point mutation as found in *M. smegmatis* has also been identified in the *inhA* of some *M. tuberculosis* strains (some of which have both *inhA* and *katG* point mutations) [82, 85], indicating that this mutation must play a role in INH resistance. Based on sequence homology and the above allelic exchange experiment, the *inhA* gene was suggested to encode a target for INH, most likely an enzyme involved in mycolic acid biosynthesis of mycobacteria, which is known to be inhibited by INH [89]. Biochemical evidence for a role of InhA in mycolic acid synthesis and its inhibition by INH has yet to be demonstrated. While mutations in *inhA* leading to alteration or amplification of target for INH can cause INH resistance [82, 85], *inhA* mutations appear to be associated with low-level INH resistance [85], in contrast to the high level of resistance caused by inactivation of catalase-peroxidase due to *katG* deletion or point mutations [81].

Because low-level INH-resistant *M. tuberculosis* strains (MIC < 5 mg/L) often still retain catalase activity [89], it was of interest to find out whether catalase-positive INH-resistant *M. tuberculosis* strains would also become sensitive to INH when transformed with *M. tuberculosis katG*. Two catalase-positive low-level INH-resistant *M. tuberculosis* strains (MIC < 5 mg/L) were transformed. Transformation of one catalase-positive INH-resistant *M. tuberculosis* strain with functional *katG* restored complete INH sensitivity, indicating that although this strain was catalase-positive it must have *katG* point mutations affecting its ability to potentiate INH activation. In contrast, another catalase-positive *M. tuberculosis* strain remained resistant to INH after *katG* transformation. The latter strain did not have *inhA* mutations either (unpublished observations). This suggests that a third mechanism besides *katG* and *inhA* mutations is responsible for INH resistance in this strain. This may be due to mutations affecting permeability or INH uptake mechanism.

Virulence of INH-resistant M. tuberculosis strains

In addition to loss of catalase activity, INH-resistant *M. tuberculosis* strains are also attenuated for virulence in guinea-pigs [88]. This attenuation of virulence is only found in INH-resistant strains but not in strains with other drug resistances. The degree of attenuation of virulence of INH-resistant *M. tuberculosis* strains for guinea-pigs is well correlated with the degree of loss of catalase-peroxidase activity [89, 94]. To confirm whether the loss of catalase activity is responsible for the attenuation of virulence of INH-resistant strains in animal models, we recently compared the virulence profiles of one *katG* deletion *M. tuberculosis* strain and the *katG* deletion strain transformed with *katG* gene expressing functional catalase activity. Preliminary data suggest that putting back the *katG* gene appears to enhance the virulence of this INH-resistant *katG* deletion strain (I. Orme, D. Young and Y. Zhang, unpublished observations). Whether INH-resistant *M. tuberculosis* strains are also attenuated for man is controversial and difficult to determine. This is because similar experiments cannot be done with human subjects and variables such as genetic factors, BCG vaccination and the body's immune status, which have important influence on the outcome of the experiment, are difficult to control. However, some indirect evidence appears to suggest that INH-resistant *M. tuberculosis* strains, especially the highly INH-resistant, catalase-negative strains, are less infective than INH-sensitive strains [94] and may also be attenuated for man. For example, 21 children, who were in close contact with a tuberculosis patient excreting highly INH-resistant tubercle bacilli, did not develop tuberculosis [94]. In contrast, the MDR-TB strain 'W' which caused an epidemic in New York is catalase positive (L. Riley, personal communication), suggesting the impor-

tance of catalase activity in the virulence of tubercle bacilli not only for animals but also for man.

Conclusions

A great deal of progress has been made on the molecular understanding of drug resistance in *M. tuberculosis* in the past few years. The identification of mutations responsible for drug resistance in *M. tuberculosis* offers new means for rapid detection of drug-resistant isolates by PCR-based techniques. It is expected that molecular understanding of drug resistance in *M. tuberculosis* will not only provide important information on the basic biology of *M. tuberculosis*, but may also shed light on the rational design of new anti-tuberculosis drugs in the future.

TUBERCULOSIS IN CHILDREN

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The recent increase in tuberculosis in adults both in industrialised and developing countries has stimulated interest in childhood tuberculosis. This paper outlines some current problems

Epidemiology

The prevalence of tuberculous infection in children is related to the number of smear-positive adults in the community [95]. Development of tuberculous disease depends on a number of factors, including age, nutrition, socio-economic circumstances, poverty, inter-current infection, the host's immune response and dose(s) of organisms.

The risk of disease in exposed children is quoted as up to 43% in those < 1 year old, 24% in children aged 1–5 years and 15% adolescents aged 11–15 years compared to 5–10% of immunologically normal adults [96]. Children are more likely to have extrapulmonary disease than adults and there is a high mortality rate, particularly in those aged 0–4 years, due to disseminated disease and meningitis [95]. However, progressive pulmonary disease is a major problem in adolescents [97–99] (Fig. 1).

In the USA the consistent decline in notifications of tuberculosis in adults and children over the decades has halted and since 1986 there has been a rise in the number of cases. There was a 35% increase in the number of notifications in children < 15 years old in 1992 compared to 1985 [100]. However, the disease remains geographically focal with two-thirds of cases reported from mainly inner cities of a small number of states. Risk factors include foreign-born children (particularly black and Hispanic groups), poverty, poor health care and exposure to adults with similar

Fig. 1. Chest X-ray of a 14-year-old boy with progressive pulmonary tuberculosis. There is loss of volume with multiple cavities and fibrosis in the right upper lobe, and a cavity in the left apex and left mid-zone. Miliary shadows are seen in both lungs. The heart is shifted to the right.

increased risk including HIV infection [96]. However, a recent estimate showed a 5.1% decrease in notifications in 1993 over 1992 in all age groups except those < 15 years, which if confirmed suggests that implementation of control strategies may be having some effect [101].

Notifications of tuberculosis in children < 15 years for 1988 show that over the decade 1978–1988 there has been a 60% decrease in annual numbers of newly notified cases and a 7.2% average annual decrease [102]. Similar to previous surveys, the rate per 10⁵ for children of Indian subcontinent (ISC) ethnic origin (29) in the 1988 survey was 20 times that for white children (1.5) and the rate in children born abroad (53) was nearly twice that of those born in the UK (26). For ISC children the rates were higher in the older age groups (10–14 years). However, since the 1988 survey there has been no decline in unconfirmed notifications and the results of the 1993 survey are awaited.

Table 3 shows the number of children with tuberculosis seen at the Royal Liverpool Children's Hospital (RLCH), Alder Hey, between 1982 and 1994 inclusive. There were in total 63 cases, 49 pulmonary and 14 extrapulmonary (with or without pulmonary disease). Fifty-four (86%) of the 63 children were white, which is in contrast to areas where a predominance of tuberculosis is in the non-white populations of the UK [103, 104]. The non-pulmonary cases comprised seven with cervical lymphadenopathy, three with meningitis and one each with hip joint, kidney, pericardium and

Table 3. Tuberculosis at the Royal Liverpool Children's Hospital, Alder Hey 1982-94

Type of infection	Number of patients			Presented to hospital	Identified by	
	Total	White	Non-white		Contact tracing	School screening
Pulmonary	49	45	4	11	35	3
Non-pulmonary*	14	9	5	12	2	0
Total	63	54	9	23	37	3

*Seven cervical lymphadenopathy, three meningitis and one each with infection of hip joint, kidney, pericardium and miliary disease.

miliary infection. Twenty-three presented with tuberculosis, 37 were the result of contact tracing and three were from the schools' screening programme, i.e., only a third of cases presented with tuberculosis without previously being identified through contact tracing or screening. The three from schools screening had minimal pulmonary lesions.

Fig. 1 shows an X-ray of severe progressive primary pulmonary tuberculosis in a 14-year-old boy. He was smear positive. A chest X-ray taken 4 years earlier showed a small node in the right hilum. Another 14-year-old boy presented with tuberculous pericarditis. No evidence of tuberculosis was detected on chest X-ray but computerised tomography (CT) showed enlarged mediastinal lymph nodes and a focus in the left lung. The value of CT in the diagnosis of primary tuberculosis in children has been described recently [105]; in 9 (60%) of 15 children with normal chest X-rays, lymphadenopathy was detected on CT.

During the same period, 31 cases of non-tuberculous mycobacterial infection were recorded including infections of cervical nodes (25), parotid (4) and skin (2). The mycobacteria identified were *M. avium-intracellulare* (16), *M. malmoense* (7) and *M. fortuitum* (1). In another three cases, non-tuberculous mycobacteria were suspected by differential antigen skin-tests. However, these 31 cases are an under-estimate as they comprise only patients referred to the TB clinic and culture-positive tissues obtained from the microbiology records. Specimens showing histological evidence of granulomatous tissue but where there was no growth are not included. Surgery with excision of the whole infected node or tissue is the best management for abscesses in the neck or other sites but this requires expertise with deep neck abscesses, particularly when there is involvement of the parotid gland with the possibility of damaging the facial nerve.

Developing countries

The incidence of tuberculosis in children in developing countries is difficult to estimate because of problems in making a diagnosis. Often the only guide is the number of children referred to tuberculosis units for treatment. A recent estimate of the incidence of tuberculosis in the 0-14-year age group from 1990 to 2000 showed that the rate per 10⁵ would increase from 41 to 142 in

Africa, whereas in all other areas, although the total number of cases would increase due to increasing population, the rate per 10⁵ would be static or fall [106]. In sub-Saharan Africa, one major cause of this increase is the co-infection of HIV and tuberculosis in the adult population. In Kampala there was a virtual doubling of hospital admissions with tuberculosis between 1985 and 1989 [107]. Although there were no overall figures for children with tuberculosis, it has been observed that there is a substantial increase in children requiring treatment for tuberculosis in Kampala (A. Okware, personal communication). Unpublished Ministry of Health statistics from Blantyre, Malawi, show that the incidence in children has risen > 10-fold between 1986 and 1993 (C.M. Parry, personal communication). In Zambia there has been an exponential rise in the number of children (as well as adults) with tuberculosis [108].

HIV infection

Children born into backgrounds with a high prevalence of adults dually infected with HIV and tuberculosis are at risk of developing tuberculosis [109, 110] even though adults, especially with more advanced HIV disease, may be less likely to transmit tuberculosis [111]. Young children with dual infection have usually contracted HIV infection perinatally and subsequently been infected by tuberculosis, whereas probably in most (but not all) dual infections in adults, tuberculosis results from reactivation of endogenous disease. Similarly to adults [112], HIV-infected children may be at greater risk of progressing from tuberculous infection to disease, although evidence for this is still lacking. Thus far, few cases of co-existing HIV infection and tuberculosis have been reported from industrialised countries [96, 113, 114]. Moss *et al.* described five children in New York co-infected with HIV and tuberculosis – four with pulmonary disease and one with meningitis [113]. *M. tuberculosis* was isolated from four of the five patients and the tuberculin test was negative in two of the three cases where it was performed; two children died.

A recent survey of 70 to 72 Pediatric Acquired Immunodeficiency Syndrome Clinical Trials Groups (PACTG) in the USA which provide care for 14 038 children, found 75 cases of children with tuberculous disease, 40 with asymptomatic infection and 71

receiving prophylaxis for tuberculous exposure [114]. The annualised case rates had increased from $58/10^5$ for PACTG sites established between 1981 and 1985 to $478/10^5$ between 1990 and 1992. The 1992 age-specific tuberculosis case rate for USA children < 5 years was $5.5/10^5$ (range 0–14) for individual states. Five (20%) of 25 isolates from PACTG patients were resistant to isoniazid and rifampicin. Eleven (15%) of 75 children failed to respond to therapy or relapsed. Children with HIV or indeterminate HIV infection are likely to be exposed to tuberculosis in the family or community. Tuberculin sensitivity may be absent or reduced and thus may not be a guide to requirement for investigation or prophylaxis [115].

Co-infection of HIV and tuberculosis in children has been reported as a problem from a number of African countries including Zaire [116], Rwanda [117], Zambia [118] and Côte d'Ivoire [119]. In Lusaka, Zambia, the seroprevalence of HIV-1 in 237 children aged 1 month–14 years hospitalised with tuberculosis during 1990–91 was 37% compared to 11% in 242 controls recruited from accident and emergency department and surgical wards. Pulmonary tuberculosis was the predominant presentation in HIV seropositive (89%) and seronegative (85%) children [118]. An effective response to a 12-month course of chemotherapy occurred in only 24% of HIV seropositive patients compared to 90% of HIV seronegative children. Of 22 (9%) children who developed hypersensitivity skin reactions (presumably to thiacetazone), 19 were HIV seropositive and three were seronegative [120]. Twelve developed Stevens-Johnson syndrome, of whom 11 [91%] died. A more recent report of 120 children with tuberculosis from the same institution, found that HIV seroprevalence had increased from 24% in 1989 to 70% in 1992 [108]. Follow up was poor (55%); however, all of the 16 patients who were known to have died were HIV seropositive. Despite exclusion of thiacetazone from the chemotherapeutic regimen, cutaneous reactions were observed in 7 (11%) of 65 children, two of whom developed fatal Stevens-Johnson syndrome. All were HIV seropositive. Miliary tuberculosis and lymphadenopathy were commoner in the HIV-seropositive compared to the HIV-seronegative group.

In Abidjan, Côte d'Ivoire, 34 (12%) of 289 children (< 15 years) with tuberculosis were HIV seropositive [119]. The highest seroprevalence (23%) was in children aged 1–4 years compared to 0.5% in a similar age group of children attending a well child clinic. HIV seropositivity was significantly associated with symptoms of wasting, chronic diarrhoea, oral candidosis and negative tuberculin skin tests. Pulmonary infiltrations and miliary disease were commoner radiological features in HIV-seropositive children.

Sixteen perinatally HIV-infected children aged 5–12 years were followed in Kigali, Rwanda [117]; four

(25%) had tuberculosis, but whether the tuberculosis was considered to be primary or reactivation was not stated.

Non-tuberculous mycobacterial infection, although less common than in HIV-infected adults, may cause problems in children [121, 122]. A review of 70 HIV-infected infants and children found 15 who had at least one *M. avium-intracellulare* complex (MAC) positive culture – seven had disseminated disease, seven had positive stool cultures and two had positive gastric aspirate cultures [122]. Disseminated MAC appeared to be related to older age groups, lower CD4 count, anergy and long-term steroid therapy.

Diagnosis

In industrialised countries the diagnosis of tuberculosis rests largely on contact tracing, tuberculin testing and chest X-ray, and thus the incidence is fairly easy to estimate. However, confirmation by sputum smear or culture is uncommon except in older children, and examination of gastric aspirates is usually reserved for problem cases, e.g., HIV-infected children with negative tuberculin tests or where drug resistance is suspected. In developing countries, immunosuppression associated with other infections and malnutrition make the diagnosis in children much more difficult as the tuberculin test is often negative or may be difficult to assess due to previous BCG immunisation; chest X-rays are often of poor quality and, therefore, difficult to interpret. Thus paediatricians often have to resort to a trial of chemotherapy.

A recent small study on induction of sputum in children in Blantyre gave encouraging results [123]. Children > 3 years old were subjected to nebulised hypertonic 3% saline. In over a quarter the diagnosis of tuberculosis could be made on sputum smear or culture.

Management

Standard chemotherapy where drug resistance is not suspected is isoniazid (INH) and rifampicin (RIF) for 6 months with pyrazinamide (PZA) for the first 2 months. If drug resistance is suspected, ethambutol or streptomycin should be added. However, ethambutol is not advised in children < 6 years old where visual acuity or colour discrimination cannot be tested. Regular intramuscular injections are now rarely given to children in industrialised countries and the requirement for daily streptomycin (SM) for 2 months is very stressful to the child (and parents).

Variations in dosage and management of selected types of tuberculosis are outlined in Table 4 [124–128]. For some time the International Union Against Tuberculosis and Lung Disease (IUATLD) has recommended 5 mg/kg/day for INH [125]. If the dose of INH is

> 10 mg/kg/day and combined with a higher dose of RIF (10–20 mg/kg/day) there is an increased risk of liver toxicity. The length of treatment for meningitis (TBM) has long been debated [129] and is exemplified in the variation outlined in Table 4. Recent reports suggest that short course therapy is adequate in uncomplicated cases, e.g., INH + RIF for 9 months [130], INH, RIF + PZA for 6 months [131], INH, RIF, PZA + SM for 6 months [132]. The outcome from TBM is mainly related to the stage of disease at presentation and promptness in instituting treatment than length of chemotherapy. Where indicated, shunt insertion is an important part of management [133].

Uncomplicated hilar lymphadenopathy with or without minimal parenchymal involvement is relatively benign and in many cases resolves spontaneously. Some authorities treat with INH + RIF for 6 months [127] or INH, RIF and PZA for 2 months followed by INH + RIF for 2 months (i.e., 4 months total duration) [126] (Table 4).

The main problems in the management of tuberculosis in children in developing countries are shortage of rifampicin – thus the necessity to give chemotherapy for up to 12 months – and HIV infection (see above). In suspected HIV infection it is advisable to replace thiacetazone with ethambutol [126], but this is not suitable for children < 6 years old who constitute the main bulk of children with tuberculosis. Reactions to thiacetazone usually present within 2–4 weeks and the child should be closely monitored during this period. Parents should be warned to report immediately pruritus and any skin reactions. It is advised that, where possible, streptomycin should be avoided in children because of the danger of HIV cross-infection through needles. The serum half-life of isoniazid and bioavailability of both isoniazid and rifampicin are higher in malnourished children and dosages may require adjustment [134].

Conclusion

Notifications of tuberculosis in children have increased in the USA since 1986, reflecting the increase in adults,

and have probably plateaued in the UK during this period. The increase is generally confined to foci in inner cities and minority groups. Major problems in the USA result from the marked increase in drug resistance.

In Liverpool, the majority of children are from the white community, which is in contrast to other areas of Britain with larger immigrant populations. The majority of cases of pulmonary tuberculosis are detected through contact tracing rather than individuals presenting with symptomatic disease. Non-tuberculous mycobacteria are a much commoner cause of cervical adenitis than that due to *M. tuberculosis* and pose clinical problems needing surgical expertise.

The epidemiology of childhood tuberculosis in developing countries, particularly sub-Saharan Africa, is strongly influenced by the prevalence of HIV infection and tuberculosis in the adult population. In the USA, co-infection with HIV and tuberculosis in children is now a problem and reflects the co-infection in the adult community. The natural history of this co-infection is not clear in children. HIV-infected children who are contacts of tuberculosis require prophylaxis irrespective of their tuberculin sensitivity.

The diagnosis of pulmonary tuberculosis remains a problem, particularly in developing countries where the tuberculin test is often negative. Induced sputum may be a practical method for diagnosis in children.

The dosages of isoniazid and rifampicin require standardisation as does duration of treatment, particularly for meningitis and other forms of extrapulmonary tuberculosis. More information is required on dosage of drugs in malnourished children. In developing countries hypersensitivity skin reactions to chemotherapy, particularly to thiacetazone in HIV-infected children, are major problems.

I thank Mrs Sue Jamieson and Mrs Ceridwen Williams (TB visitors) for assistance in compiling the Liverpool paediatric tuberculosis data and Dr J. L. Stanford, University College and Middlesex School of Medicine, for supplying non-tuberculous mycobacterial antigens.

Table 4. Management — variations in children

Source	Dose (mg/kg)		Duration of therapy (months) for		
	INH	RIF	Tuberculous meningitis	Bones/joints	Hilar lymphadenopathy†
Joint TB Committee (1990) [124]	10	10	12	9(6)	6
IUATLD (1991) [125]	5	10–20	9	6	6
WHO (1993) [126]	5	10	9	9*	4
Amer Acad Red (1992) [127]	10–15	10–20	12(6–9)	12(6–9)	6‡
CD (1993) [128]	10–20	10–20	12	12	...

(), Possible reduced duration.

*Bone disease with neurological complications.

†With minimal parenchymal involvement.

‡INH + RIF only for 6 months.

ADULT TUBERCULOSIS

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Introduction

Adult tuberculosis is a disease of two worlds; no longer is it strictly divisible into developed and developing countries but rather by ethnic differences. The white population of western Europe, America and Australasia have very low rates of disease between 5 and 15/10⁵/annum. Disease is seen most frequently in the elderly where recrudescence of infection acquired perhaps decades previously is probably the principal cause of disease.

Among ethnic minority groups in developed countries, rates are nearer 100/10⁵/annum and disease is as common in young adults as it was in the white population of Europe 50 years ago. Within the developing countries of Africa and Asia, rates of disease exceed 100/10⁵ and may be as high as 200/10⁵ in some areas such as the Philippines. To this worrying scenario is added the further complication of HIV. Dual infection with HIV and the tubercle bacillus considerably increases the likelihood of infection leading to disease from *c.* 10% in a lifetime to 10% per annum. In developing countries where the

incidence of infection is already high, in young adults the advent of HIV has resulted in rates of disease exceeding 1000/10⁵/annum [15, 135].

Clinical presentation

The characteristic feature of the clinical presentation of tuberculosis in adults is chronicity. Patients characteristically present with symptoms of several months duration and these are, for pulmonary disease, in decreasing order of frequency: cough (often with haemoptysis), weight loss, malaise, breathlessness and, more rarely, pain. Physical examination may be remarkably unrewarding and in relatively advanced disease often presents with few or no abnormal features. However, the patient may appear wasted and a dry irritable cough may sometimes be present. In advanced disease, evidence of pneumonic changes in the lungs with bronchial breathing and crepitations usually in the upper zones may be heard. If disease is extensive, upper lobe destruction with amphoric breathing may occur. If the disease is of long standing, fibrosis with resultant deviation of the trachea may be present (Figs. 2–7).

In the UK, 5–10% of patients with active disease are diagnosed on contact screening and may be asymptomatic. In adults, *c.* 80% of chest X-rays show the characteristic changes of tuberculosis, that is upper lobe disease, although in 5–10% the apical segment of the lower lobe may be affected. The shadowing is soft floccular-nodular with cavitation in 10–50% of cases.

Fig. 2. Chest X-ray of a 70-year-old white man with sputum smear-positive tuberculosis. The patient had had pulmonary tuberculosis 15 years earlier. Note the well-defined linear shadows in the left apex indicative of fibrotic healing of old disease. In addition there is soft floccular shadowing with cavitation characteristic of active disease.

Fig. 3. Chest X-ray of a 40-year-old white man with sputum smear-positive tuberculosis. Comparison with Fig. 2 shows the absence of fibrotic shadows, and evidence of new disease only. This patient was the son of the patient in Fig. 2.

Fig. 4. Chest X-ray of a 29-year-old Indonesian woman showing a large cavity in the left upper lobe.

Fig. 6. Chest X-ray of a patient with extensive pulmonary tuberculosis with total destruction of the right upper lobe. The patient died after 1 week of treatment.

Fig. 5. Chest X-ray of a 60-year-old woman with chronic active disease. Note the calcified lesion in the left upper lobe and deviation of the trachea to the left.

Often the chest X-ray may appear virtually diagnostic but the differential diagnosis should include infection with other bacteria such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, other *Mycobacterium* spp., organising pneumonia, rheumatoid lung, progressive massive fibrosis, occupational disease, autoimmune disease and most commonly in the developed world, carcinoma of the bronchus. Tuberculosis, if suspected,

Fig. 7. Chest X-ray showing miliary tuberculosis.

should be confirmed by sputum examination, by smear and culture for acid-fast bacilli. A positive smear is virtually diagnostic of tuberculosis, but increasingly the elderly living in developed countries may be affected by non-tuberculous mycobacteria such as *M. kansasii* or *M. xenopi*. Only culture of *M. tuberculosis* confirms the diagnosis. In practice, only 50–60% of pulmonary tuberculosis cases notified in the UK are sputum smear-positive and 60–70% confirmed on culture. The remainder is assumed to be tuberculosis on clinical grounds and by response to treatment.

If the patient is not able to produce adequate specimens of sputum spontaneously, further efforts should be made to obtain sputum by other means such as sputum induction, transtracheal injection of nebulised saline, transtracheal aspiration or bronchoscopy with lavage. Bronchoscopy is very useful in helping to exclude differential diagnoses such as malignancy and may be employed routinely in developed countries where it is easily accessible [136].

Non-respiratory disease

In the white population, a non-respiratory site accounts for 15% of cases. In the ethnic minority groups in the UK up to 50% of patients may present with a non-respiratory site of infection. These are in order of frequency: cervical lymph nodes, bone and joint disease, abscesses of the soft tissues, genito-urinary (GU) disease, abdominal disease, meningeal and miliary tuberculosis. GU disease is more frequent in the older white population. The clinical presentation again is chronic. Lymph nodes may appear enlarged and rubbery and sometimes tender, and they may occasionally suppurate causing chronic sinus discharge. An abscess will present as a fluctuant swelling. Any site may be affected but the commonest site for an abscess is the inguinal region, as pus tracks from a spinal focus of infection.

Half of all bone and joint disease is spinal with hip and knee infections occurring next most commonly. GU disease in the female may present as infertility or chronic low abdominal pain. In men, painful epididymal swelling is the commonest presentation. Meningeal disease may present clinically with gradual loss of function of a cranial nerve, characteristically the 4th, 6th or 8th. Alternatively, the disease may present more acutely with gastric signs and symptoms of meningitis. The golden rule must be to collect appropriate specimens for smear and culture before treatment is commenced [137]. Modern molecular techniques currently being developed may be of use in diagnosis in the future.

Treatment

All forms of tuberculosis, with the exception of tuberculous meningitis, may be treated with isoniazid,

rifampicin and pyrazinamide for 2 months followed by isoniazid and rifampicin for 4 months, provided that the mycobacteria are fully sensitive. For tuberculous meningitis, the two-drug phase should be continued for a further 10 months and streptomycin is often given in the initial 2-month intensive phase as an additional fourth drug. If there is a possibility of drug resistance, a fourth drug (either ethambutol or streptomycin) should be added to the initial phase. Intermittent three times weekly directly observed therapy is a useful option and should be used where there may be any doubt as to whether the patient is complying with treatment [138].

Specimens should always be sent for susceptibility testing as well as culture. Usually these are available 6 weeks after specimens have been sent and it is probably prudent to continue treatment with three or four drugs until the clinician can be sure that the isolate is fully susceptible to the drugs that the patient is taking.

Drug resistance

Drug resistance has always been a feature of tuberculosis treatment since streptomycin was first used in 1944. In more recent times it poses a new threat as bacilli resistant to isoniazid and rifampicin are emerging. These are particularly common among those who have had previous treatment, ethnic minority groups, those migrating from certain developing countries, patients who are HIV-positive, substance abusers and the homeless. Any patients in whom the diagnosis of tuberculosis is suspected and who fall into one of these categories, should be treated with at least four drugs until drug susceptibilities are known. If a clear history of previous drugs taken for tuberculosis chemotherapy can be determined at least two and preferably three drugs not previously used should be prescribed. The 'second-line' therapeutic agents available to treat tuberculosis are shown in Table 5 [139].

New techniques in the management of tuberculosis

Molecular biological techniques potentially offer rapid methods of diagnosis and susceptibility testing. The time taken for traditional methods of confirming the presence of *M. tuberculosis* in a specimen ranged from 3 to 6 weeks on Lowenstein-Jensen medium. Even with radiometric technology (Bactec; Becton Dickinson), 2–3 weeks may be required for culture confirmation and a further week for susceptibility testing. Methods based upon the polymerase chain reaction (PCR) for amplification of the mycobacterial genome enable identification of the organism within hours of the specimen being received [140]. The use of restriction fragment-length polymorphism (RFLP) may make it possible to compare apparently identical strains of *M. tuberculosis* from different individuals by DNA fingerprinting [141] and, therefore, provide a means of quick susceptibility

Table 5. Second line drugs in the treatment of tuberculosis

Old drugs	Newer drugs
Ethionamide	Quinolones, e.g., ciprofloxacin
Prethionamide	Clarithromycin
Cycloserine	Azithromycin
Amikacin	Clofazamine
Kanamycin	Rifabutin [139]
Para-amino-salicylic acid (PAS)	

determination if the susceptibility of the initial strain is known.

Central laboratory testing

The international threat of drug-resistant tuberculosis resulting in incurable disease is a very real prospect. Ideally, for patients likely to have a drug-resistant organism (see above) who are found to be sputum smear positive on local laboratory testing, specimens should be sent to a central laboratory where PCR testing could identify the organism within hours. The provision of a national central bank of DNA fingerprinting raises the possibility of rapid susceptibility determination by DNA comparison within hours once a sufficiently large 'bank' of 'fingerprints' is established. If an isolate produces a fingerprint found to be identical with another which, on routine testing, has been found to be resistant to one or more drugs, the clinician can be informed within hours and an appropriate drug regimen started.

Central laboratory provision would require central funding, but the cost and prevention of potential drug resistance problems would be small compared with the eventual cost of failing to control drug-resistant tuberculosis.

Conclusions

Tuberculosis is now a relatively rare disease in the developed world. Relatively few clinicians will have experience of patient management or use of anti-tuberculous medication. The management of patients should therefore remain in the hands of the relatively few trained experts, who usually have a background of chest medicine or infectious diseases. The provision of a central laboratory for PCR and RFLP analysis and referencing should be made a national priority.

TUBERCULOSIS AND HIV INFECTION

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Introduction

In the last few years most Western industrialised countries have experienced a startling increase in the

incidence of tuberculosis (TB). This unprecedented change is the reversal of a gradual trend that has been in operation for decades [142]. Whilst several factors, such as the running down of national tuberculosis control programmes and immigration from endemic areas, have contributed to the upsurge in the number of cases of tuberculosis, the single most important issue has undoubtedly been the sudden emergence of HIV infection [143].

Whilst the interaction between HIV infection and tuberculosis has been most apparent in the rich industrialised countries, the greatest impact has been in sub-Saharan Africa where HIV infection has penetrated widely and tuberculosis is highly prevalent [144]. As the HIV epidemic spreads rapidly in Asia it is likely that an even greater problem will occur in the Indian subcontinent in the next decade.

Much has been written about HIV and tuberculosis and several comprehensive reviews are recommended [112, 143–145]. This article will focus on three aspects of tuberculosis-HIV co-infection.

Mechanism of interaction

The exact way(s) that the progressive immunosuppression caused directly by HIV infection alters host immunity to tuberculosis are not well understood. It appears that HIV increases the susceptibility to infection and disease in naive individuals following exposure, as shown in several nosocomial outbreaks of tuberculosis in AIDS-care facilities in the USA [62]. It also appears that for individuals who are already infected with tuberculosis (as indicated by positive PPD skin testing), co-infection with HIV markedly increases the risk of endogenous tuberculosis reactivation [146].

In areas that are endemic for tuberculosis it is not clear which process is more important, and whether most cases are the result of acute infection (or re-infection where prior exposure has occurred) or represent the reactivation of an endogenous focus. Clearly, both could be occurring. It has been assumed widely that most cases were reactivation in the same way that most cases of adult tuberculosis before the advent of HIV were considered to be reactivation or post-primary disease [147].

Modern molecular techniques such as DNA fingerprinting are making important contributions to studies of tuberculosis epidemiology. Recent studies from New York [148] and San Francisco [149] have concluded that at least one-third of new cases result from recent person-to-person transmission whereas it was predicted that nearly all would be reactivation. In Kenya we have similar unpublished results from a cohort of HIV-infected women: DNA fingerprinting revealed that at least 25% of tuberculosis disease was the result of

recent infection or re-infection, whereas the expectation was that nearly all cases would be reactivated disease.

There are several reasons why it is important to clarify whether most HIV-tuberculosis is new or recurrent disease in areas endemic for tuberculosis. Chemoprophylaxis will be of use to help prevent active disease in adults if reactivation is the dominant process, but if most cases are acute new infections then prophylaxis will be relatively ineffective [150]. Immunosuppressed patients will be at risk if exposed to tuberculosis regardless of whether they have been exposed previously as children. Nosocomial transmission is thus likely to be a major problem. In contact tracing, an adult with active pulmonary disease may now be as likely to be a new case as the index case. Finally, relapses after standard therapy may represent re-infection rather than treatment failure, arguing against longer anti-tuberculosis treatment in HIV-tuberculosis.

Clinical spectrum of HIV-tuberculosis

M. tuberculosis is a virulent pathogen that can cause disease at nearly all stages of HIV infection. There is a wide spectrum of clinical disease in adults which ranges from typical pulmonary presentation, in those with relatively well preserved immunity and CD4 counts in excess of 200 cells/mm³, to atypical disseminated disease in markedly immunosuppressed individuals [151]. Mycobacteraemia may often occur in the latter patient group [152].

In adults with pulmonary disease, cavity formation occurs less frequently and there is much less predilection for the apical regions. Unusual lower lobe infiltrates which may be bilateral and are smear negative but culture positive are the commonest diagnostic challenge. Without bronchoscopy or induced sputum, many diagnoses are established by a 1-month therapeutic trial. Pleural effusions occur more frequently with HIV infection and can sometimes be bilateral [153].

Overall, extrapulmonary disease is much more common. In some series up to half the HIV-tuberculosis is extrapulmonary compared to only *c.* 10% in immunocompetent individuals. The most frequent presentation is lymphadenopathy. Other important presentations are pericardial disease, meningitis and miliary disease. Disseminated mycobacteraemia with little host response is being recognised increasingly and is an important cause of, or terminal event in, slim disease (chronic diarrhoea and wasting) [154].

The spectrum of clinical disease seen is much more suggestive of primary tuberculosis than adult post-primary reactivation disease, again suggesting that acute (re)infection is an important mechanism of disease. Much less is known about the spectrum of paediatric HIV-tuberculosis because of the difficulties

of diagnosis in areas where it would be expected to be a relatively common problem.

The response to standard treatment appears adequate, at least in the earlier stages of HIV infection. The high mortality seen in endemic areas during therapy is usually the result of other severe bacterial infections such as pneumococcal pneumonia or disseminated nontyphi salmonellosis, rather than a failure to respond to appropriate therapy [155]. Given the widespread and heterogeneous pattern of drug resistance, it is sensible to obtain drug susceptibility profiles on initial isolates. In most parts of the developing world this is not possible as a routine investigation and national recommendations have to be used.

Another difficulty of treating HIV-tuberculosis in resource-poor countries is the widespread routine use of thiacetazone as a first line anti-tuberculosis drug. This drug is toxic in individuals with underlying HIV infection and can cause hypersensitivity reactions and severe Stevens-Johnson syndrome in up to 20% of cases [156]. The cost implications of replacing thiacetazone with more expensive short-course regimens containing rifampicin are significant and many African countries cannot afford to switch even though this is a much safer policy [157].

Public health implications

Individuals who are sputum smear positive are highly infectious and the main reservoir of infection. As already pointed out, adults with HIV-tuberculosis are much more likely to have extrapulmonary disease, which is essentially non-infectious. If the lungs are involved the patient is much less likely to have open cavitating disease, be strongly smear positive, and is more likely to have smear-negative disease with diffuse lower lobe shadowing. Overall, an index case with underlying HIV infection will pose less of a public health threat than a seronegative index case.

In communities where tuberculosis is a rare disease and there are very few potentially infectious cases, any person who is sputum culture positive will be a potential source of infection. This is of particular importance in hospitals or care centres where other immunosuppressed patients may be in close proximity. In communities where there is significant tuberculosis transmission most will be exposed as children or young adults. Having more individuals of relatively low infectiousness will hardly alter transmission dynamics. However, there is still an important and significant risk of nosocomial transmission.

The main difficulty in industrialised countries is in identifying the patient with tuberculosis as soon as possible in order to minimise the threat to public health. There is no substitute for increased clinical vigilance. In developing countries the single dominant

issue is how to provide enough resources to continue to run a tuberculosis control programme when faced with a doubling or even trebling of the demand for services caused entirely by HIV co-infection. In some areas services are starting to collapse under the weight of new cases, and the existing tuberculosis and TB-HIV problem is worsening dramatically.

Conclusions

There is little to be complacent or optimistic about when considering the impact that HIV has had and increasingly will have on tuberculosis. The impact is already devastating and in some areas totally disrupting existing tuberculosis programmes. The burden of disease is increasing everywhere.

However, in taking a global view one point must be emphasised. Whilst the global burden of tuberculosis will rise over the next few years, much of the actual increase in the overall cases of tuberculosis is demographic, arising from a larger population who will become infected. It is estimated that in 1999 only 14% of tuberculosis cases and deaths will occur worldwide in individuals co-infected with HIV [158]. It may be relatively short-sighted and counter-productive constantly to present tuberculosis as an HIV-associated problem, because it may become considered as a problem of HIV infection alone.

The HIV epidemic has cruelly exposed weaknesses in tuberculosis control worldwide and is undoubtedly making them worse. However, the HIV epidemic did not cause them. If the medical community has learnt nothing else in the last few years it must be that tuberculosis remains a global priority even without the interaction with HIV [159].

TUBERCULOSIS — NEW ASPECTS OF CHEMOTHERAPY

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Although man has been aware of tuberculosis, as a disease, for many years, it was only around a century ago that the causative agent was isolated and for only less than half a century has man been able to treat this devastating disease. We are now in the era of multi-drug-resistant variants of *M. tuberculosis*; these organ-

isms, combined with victims whose immune systems are frequently compromised or destroyed, now present modern medicine with a new set of dilemmas. However, it is not just these new 'doomsday' organisms which pose a threat, it is the regular availability of standard drugs, the affordability of these agents to developing countries and compliance by infected patients with the lengthy regimens necessary for the eradication of one of the most common infections on the earth.

It is worth recounting the brief history of efforts to control tuberculosis. Streptomycin was introduced in 1946 [160]; however, it was soon recognised that isolates rapidly became resistant to the drug. This was disappointing, as early clinical improvement soon gave way to deterioration [160]. The recognition of isonicotinic acid, or isoniazid, allowed combination therapy to be initiated in 1952 [161]. Later in the 1950s the addition of para-aminosalicylic acid (PAS) to this dual therapy allowed long courses of chemotherapy to be studied by the British Medical Research Council [162]. These courses lasted for 18–24 months and they remained the 'norm' for 20 years until studies from East Africa showed that the addition of rifampicin enabled shorter courses of 6 months therapy to be introduced. These regimens included rifampicin, isoniazid and streptomycin [163–165].

Curiously, studies conducted in 1956 [166] showed pyrazinamide to be active within the macrophage but this agent was not included in anti-tuberculous regimens until the mid-1970s [167]. Two further studies demonstrated that the addition of pyrazinamide for 2 months enhanced the 6-month course of isoniazid and rifampicin [168, 169]. Current efforts are being focused on the use of isoniazid alone as a prophylactic agent [170]; however, the drive towards new additions to the established recommended regimens (Table 6) is motivated by the need to find agents active against susceptible and multiresistant strains of *M. tuberculosis*, to provide therapies which can be given less frequently, have fewer side effects and can be readily available throughout the world at an affordable cost. Clearly, no single agent will fulfil all of these requirements, but the drive for a new agent encompasses the established classes of drugs and new chemical entities.

The mode of action of each of the established and potential anti-tuberculous agents is shown in Table 7.

Table 6. Current recommendations for tuberculosis therapy and associated side-effects

Drug	Dosage	Maximum daily dose	Side-effects
Isoniazid	5 mg/kg p.o./i.m.	300 mg	Hepatic enzymes, neuropathy, hepatitis, neuritis, hypersensitivity rash
Rifampicin	10 mg/kg p.o.	600 mg	Orange discolouration of urine and secretions, nausea, vomiting, hepatitis,
Pyrazinamide	15–30 mg/kg p.o.	2 g	Hepatotoxicity, hyperuricaemia
Ethambutol	15–25 mg/kg p.o.	2.5 g	Optic neuritis, skin rash, visual acuity

i.m., Intra-muscular; p.o., by mouth.

Table 7. Mode of action of current and potential anti-tuberculosis agents

Drug	Target site
Isoniazid	Acts on cell-wall mycolic acid synthesis Acts on nicotinamide metabolism
Rifampicin	Inhibits RNA polymerase and RNA synthesis
Ethambutol	Acts on polyamine function Acts on cell-wall synthesis
Pyrazinamide	Mechanism unknown
Oxazolidinones	Protein synthesis inhibitors
Gangamicin	Cell-wall synthesis inhibitor
Acridinones	RNA inhibitors
Quinolones	DNA synthesis inhibitors

The only group which targets a new site of action comprises the fluoroquinolones, since both the established and other new agents are targeted on cell-wall synthesis, protein synthesis and RNA synthesis. A brief resumé of the mechanism of action of the current agents is given below.

Isoniazid is the most active drug against *M. tuberculosis*, whilst retaining a relatively safe profile. It is bactericidal, but is active only against replicating bacteria [171]. However, the spontaneous mutation rate to isoniazid resistance is in the range 10^{25} – 10^{26} . Isoniazid is thought to inhibit the synthesis of mycolic acids which are integral for the structure of the mycobacterial cell wall [172]. Additionally, isoniazid may interfere with the metabolism of nicotinamide (NAD) by the production of the isoniazid metabolite, isonicotinic acid [173]. Clearly, this competitive role affects reactions which are catalysed by NAD- and NADP-dependent dehydrogenases [174].

Rifampicin and its derivatives the rifamycins (e.g., rifabutin and rifapentine) are powerful, specific inhibitors of RNA polymerase. This enzyme is intimately involved in RNA synthesis. Rifampicin binds to the β subunit which acts as the catalytic centre of the enzyme, and thus inhibits the initiation of RNA synthesis [171].

Ethambutol possesses activity against *M. tuberculosis*, but is less active against other mycobacterial species. It is not active against sessile cells and less potent *versus* rapidly growing bacteria. Two target sites have been elucidated, polyamine function and cell-wall synthesis. It was shown that ethambutol was a specific inhibitor of spermidine synthase [175]. However, the primary mechanism of action of ethambutol is the inhibition of arabinogalactan synthesis, which is critical in the development of cell-wall structure [176].

Pyrazinamide, when used in combination with isoniazid, is rapidly bactericidal for *M. tuberculosis* but has no action against other mycobacterial species [177]. The activity is dependent upon conversion of pyrazinamide to pyrazinoic acid, which occurs at pH 5.6. Exactly how this agent acts so specifically on *M.*

tuberculosis is not known, but it may be due to the in-vivo synergic combination of hormone-stimulated macrophage activity and pyrazinoic acid.

Oxazolidinones are a new class of specific bacterial protein synthesis inhibitors and exert activity against *M. tuberculosis*, both methicillin-susceptible and -resistant *Staphylococcus aureus* and other gram-positive bacteria [178, 179]. Interestingly, these agents have no activity against *M. avium*.

Gangamicin is a new synthetic antibiotic (also known as azaquinone), which exerts its activity by virtue of being an analogue of co-enzyme Q and vitamin K and thereby inhibits cell-wall synthesis [180].

Acridinones are a recent introduction and their mode of action may involve RNA synthesis inhibition [181].

Quinolones act specifically upon prokaryotic DNA gyrase, which is involved in DNA replication controlling DNA unwinding and super-coiling following DNA replication [182].

In the intervening 40 years since the initial clinical studies in tuberculosis chemotherapy, many significant adaptations of the management of patients have occurred. The previously regarded supportive measures of hospitalisation, isolation, diet, rest and surgery have all been shown to have little significance in the management of tuberculosis [183].

One of the most significant steps towards improving and rationalising new therapies for tuberculosis is adoption of the guidelines created by the US Food and Drug Administration and the Infectious Disease Society of America (FDA/IDSA) working group. The guidelines for the evaluation of new anti-mycobacterial agents were published in 1992 [183]. In essence, the document covers the following key components or stages of drug evaluation: (i) in-vitro studies; (ii) in-vivo studies — inclusion criteria for trials, exclusion criteria for trials, study design, response to therapy, safety assessments. The key features of these phases of development are summarised in Tables 8 and 9. However, it is clear that to date few of these new guidelines have been applied to the new, potentially useful agents or classes of drug. The reasons for these omissions will be addressed later. Nevertheless, there are several candidate drugs which are being nominated as potential saviours in the battle against tuberculosis.

The most logical agents to be pursued in this new fight are the rifamycins such as rifabutin or rifapentine; other examples would include KRM-1648 [184, 185] from Kaneka and SPA-S-565 [186] which is being jointly developed by SPA and Glaxo. Rifabutin was the first new anti-mycobacterial agent to be approved for 20 years. It has better activity than rifampicin

Table 8. Key stages of new drug development for anti-tuberculosis therapy [183]*In vitro*

- assessment of study drug only and in combination with likely agent at various pH values
- evaluation of drug's activity against *M. tuberculosis* in macrophages
- estimation of rate of mutation

In vivo

- evaluate potential drug interactions
- assess effect of the study agent on microbial flora
- examine the host normal flora for potential resistance development
- determine the activity in appropriate animal model
- ascertain whether drug is bactericidal or bacteristatic
- assess the long-term effects in terms of survival or relapse

Table 9. Key criteria and assessments in clinical trial design [183]

What is the study drug intended to do:

- reduce duration of therapy (frequency)
- reduce toxicity
- act on resistant strains
- improve compliance

Study must be comparative, randomised, preferably double-blind, may be stratified

Response to therapy — microbiological outcome is KEY

Therapy failure is clearly defined

Follow-up must be adequate

- in HIV-negative patients 5 years
- in HIV-positive patients 2–3 years

Safety — include ALL major organs and senses e.g.:

- cardiovascular alterations
- ocular disturbance
- gastrointestinal changes

against *M. avium* as well as being more active than rifampicin against *M. tuberculosis*. It is active against 30% of rifampicin-resistant strains of *M. tuberculosis*. Rifabutin has less potential for drug interactions than rifampicin, as it does not induce liver enzymes to the same degree. Rifabutin also has a longer half-life than rifampicin (32–67 h *versus* 3.3 h). Thus, it is possible to give the drug less frequently, particularly for prophylaxis [187]. Rifapentine is in phase II studies in various countries based largely on its 10-fold greater activity against *M. tuberculosis* compared to rifampicin. However, unlike rifabutin, it is not active against rifampicin-resistant strains. Again, a longer half-life may allow intermittent therapeutic application [188].

The first of a new group, the benzoxazino-rifampicins, KRM-1648, is in pre-clinical and early phase I development. Evidence so far suggests excellent activity against *M. tuberculosis* and *M. avium*, MIC₉₀s are < 0.0125 mg/L and 0.05–0.1 mg/L, respectively. Rat pharmacokinetic studies show a long elimination half-life and excellent tissue distribution; however, it is highly protein bound. In animal models, KRM-1648

reduced lung lesions and bacterial load as well as increasing survival times [185].

A totally new class of antibacterial agent currently under development comprises the oxazolidinones, which are potent specific inhibitors of bacterial protein synthesis. These compounds are active against mycobacteria as well as gram-positive bacteria such as methicillin-resistant staphylococci. U-97456 (Fig. 8) is one of a series of five indolyl derivatives which has an MIC of 1 mg/L against *M. tuberculosis*; however, it has no activity against *M. avium* [178].

By targeting a unique bacterial cellular target, the cell wall, another new synthesis inhibitor has been patented for development. Azaquinone or gangamicin (BM-40501) was synthesised by Dr P. Gangadharam of Chicago. It is an analogue of the ubiquinone (co-enzyme Q 10) and acts as an inhibitor of the cell-wall synthetic process [180]. Azaquinone is active against both *M. tuberculosis* and *M. avium*, (MICs 1–20 mg/L and 2.0–8.0 mg/L, respectively). The activity also encompasses drug-resistant *M. tuberculosis* strains. In murine models it showed activity in spleen and lung lesions [180].

Before examining the most controversial new group, the quinolones, there are three other potentially useful compounds that deserve mention. The 2'2' bipyridyl analogs, VUF 8514 and VUF 8842, have bactericidal activity against *M. tuberculosis*, including resistant strains [189]. K-130 is a dihydrofolate reductase inhibitor, which although being targeted towards *M. leprae* does possess activity against *M. tuberculosis* [190]. BCH 950 is an inhibitor of cell-wall synthesis similar to isoniazid but with fewer adverse effects. It is in phase I clinical trials [191].

The fluoroquinolones, derived from nalidixic acid, have been postulated as major additions to the anti-tuberculosis armamentarium for almost 10 years. Many of the currently available and developmental fluorinated quinolones possess anti-mycobacterial activity (Table 10) [192–196]. In addition to a broad spectrum of activity, the other features of the fluoroquinolones include good absorption after oral administration and a large volume of distribution into tissues and fluids, including those of the respiratory tree [197]. Perhaps most significantly, quinolones are taken into macrophages and retain their activity intracellularly [198]. A

Fig. 8. Structure of a new protein synthesis inhibitor U-97456, oxazolidinone.

Table 10. Activities of fluoroquinolones against *M. tuberculosis*

Agent	MIC90 (mg/L)	References
Norfloxacin	2.0	192
Pefloxacin	2.0	192
Ciprofloxacin	0.5	192
Ofloxacin	0.5–0.78	192, 193, 194
Sparfloxacin	0.2	193
Clinafloxacin	0.25	194
OPC 17116	3.13	195
Du 6859a	0.25	196

useful feature is that, as the site of action of the quinolones is different from all the other main anti-tuberculosis agents, cross-resistance should not be a problem [199]. Other advantages of the use of quinolones as part of a multi-drug anti-tuberculosis regimen include a good safety profile and, so far, acquired development of resistance has not been seen in many mycobacterial infections. However, the major drawback of quinolone usage, particularly in the developing countries, is the cost of the drugs.

Ciprofloxacin has been used as part of combination therapy for both tuberculous and non-tuberculous mycobacterial infections. The clinical use of ciprofloxacin was based on favourable pharmacokinetics [200], good activity against *M. tuberculosis* [192] and evidence from animal model studies [201]. These data prompted use of ciprofloxacin in various regimens against drug-susceptible and -resistant strains. Anagnostopula [202] reported nine cases of multi-drug-resistant *M. tuberculosis* which were treated with isoniazid, ciprofloxacin and other non-stated agents for up to 4 months. The patients were treated for 12 months and followed for a further 12 months. No relapses were observed and sputum clearance occurred in seven of nine patients by the fifth month; the other two patients improved clinically but did not clear their sputum. Kahana and Spino [203] used ciprofloxacin in various regimens in 15 patients with mycobacterial infections, of which 11 had tuberculosis. Each of the patients' isolates were either resistant (*in vitro*) to one or more of the first line agents or were showing no clinical response. Ciprofloxacin was given at a dose of 500–750 mg daily with either rifampicin or rifabutin in 12 cases; the three other patients received a variety of

agents concomitantly. Ten of the patients were evaluable 12 months after therapy ceased; in seven of these a clinical improvement was observed. The side effects were typical of both agents used, i.e., nausea (four patients), crystalluria (one), leukopenia (one) and renal impairment in one which ceased on termination of ciprofloxacin therapy. Pharmacokinetic studies conducted within the study indicated that favourable serum levels were achieved with once daily dosing. A further 65 patients were randomised by Mohanty and Dhamgaye [204] to receive either streptomycin, isoniazid, rifampicin and pyrazinamide (A) or streptomycin, isoniazid, ciprofloxacin and pyrazinamide (B). After 6 months, sputum smear results were negative in all of 17 patients who had received regimen A, whilst 17 of 18 were negative who received regimen B. Two years after stopping therapy, there was one relapse in group A and three in group B. The incidence of adverse events was similar in both groups.

Concurrent with these reports, a group from the Royal Free Hospital, London, and the Kilimanjaro Christian Medical Centre, Moshi, Tanzania, undertook a large-scale, randomised, comparative study following earlier laboratory and clinical research that indicated that ciprofloxacin, 750 mg daily for 7 days, had a bactericidal activity comparable to that of isoniazid, 300 mg [205]. Investigations into the sterilising activity of a combination of ciprofloxacin, isoniazid and rifampicin showed it to be less than that of isoniazid, rifampicin, pyrazinamide and ethambutol (Table 11). The preliminary results from the large-scale study indicated that in HIV-negative patients there was little difference between the two groups; however, in HIV-positive patients the ciprofloxacin-containing regimen was less effective in terms of sputum smear clearance and relapses after termination of therapy [205]. The investigators suggest that an alternative regimen be evaluated, probably ciprofloxacin, rifampicin and pyrazinamide, which retained the features of the latter agent with the better safety profile of the quinolone. One of the most significant findings of the recently completed study was that none of the isolates from patients who were treated with the ciprofloxacin-containing regimen developed resistance to the quinolone. Indeed, no quinolone-resistant strains were

Table 11. Sterilising activity of regimens with and without ciprofloxacin [205]

Criterion	Regimen A (11)	Regimen B (9)
Culture negative at 4 weeks	9/10 (90%)	3/9 (33%)*
Culture negative at 8 weeks	11/11 (100%)	6/9 (67%)†
Number of weeks culture negative by end of study (8 weeks)		
Mean	4.7	2.7‡
Median	5	2
Range	2–7	0–7

Regimen A, isoniazid, rifampicin, pyrazinamide, ethambutol (11 patients); regimen B, isoniazid, rifampicin, ciprofloxacin (9 patients).

* $p = 0.02$, † $p = 0.07$ Fisher's exact test. ‡ $p = 0.11$, Wilcoxon rank sum test.

isolated at any time during the study (S.H. Gillespie, personal communication).

Ofloxacin has an anti-mycobacterial spectrum similar to that of ciprofloxacin [206]. The bio-availability of ofloxacin is 90–98%, compared with 80–90% for ciprofloxacin [207]. Ofloxacin has a slightly longer half-life and it too has been suggested as a potential anti-mycobacterial agent. The MIC of ofloxacin for *M. tuberculosis* is 1.4 mg/L compared with 0.48 mg/L for ciprofloxacin [208]. However, in-vitro studies, such as animal models, were not conducted for ofloxacin until 1991 [209], whereupon the dose-related effect of the drug was observed. The minimum effective dose was 150 mg/day, but 300 mg displayed better therapeutic effects.

Prior to these animal studies, Tsukamura *et al.* [210] examined ofloxacin either alone or in combination with other drugs in the treatment of multi-drug-resistant or long-term culture-positive *M. tuberculosis* infections. Sputum cultures in five of 19 patients treated converted, but resistance of ofloxacin occurred in 12 of the other cases. An ofloxacin dose of 300 mg/day was administered. The issue of dosing with ofloxacin has been the subject of controversy. Davidson and Lee [211] report having used 400 mg/day, Truffot-Pernet [209] stated that 600 mg/day may be only moderately effective, whilst Cambau *et al.* [212] reported a case of ofloxacin resistance developing in a patient given 800 mg/day. The latter, given as a single dose, is not licensed in many countries as it seems likely to lead to considerable central nervous system side-effects. Perhaps 400 mg twice a day may be better tolerated, but possibly with decreased compliance.

The ofloxacin resistance issue raised by Cambau *et al.* [212] was found to be a single point mutation at amino-acid position 87 of the DNA gyrase A sequence. A shift from aspartic acid to histidine conferred an MIC change from 1 mg/L to 32 mg/L. It seems curious that this type of change has been seen with ofloxacin [210, 212] and yet is rarely observed with ciprofloxacin.

As the data for the first of the fluorinated quinolones – ciprofloxacin and ofloxacin – are not truly convincing, the issues of dose, duration of therapy and concomitant agents are still to be resolved. Thus, it is to the newer quinolones we turn, such as sparfloxacin (Dainippon/Rhone-Poulenc-Rorer), OPC-17116 (Otsuka), Du 6859a (Daiichi), clinafoxacin (Parke-Davis/Bayer) and other agents still in the pre-clinical and early phases of development. Sparfloxacin is registered in Japan (1993) and France (1994) with other countries expected soon. It has been developed with community respiratory tract infections in mind; this, coupled with a favourable MIC for *M. tuberculosis*, have suggested that it may have a role in the treatment of tuberculosis. However, there are few published data on animal model studies

or even modest numbers of clinical case reports. Certainly its activity and long elimination half-life may be optimal; however, recent reports of photosensitivity from the Japanese Department of Health [213] which confirmed 53 cases reported within the first year of general use in Japan, suggest that, as with temafloxacin (now withdrawn worldwide), advantages may also be accompanied by disadvantages. There is a need for further specific toxicological work with the newer quinolones before large scale tuberculosis treatment studies can be undertaken.

It is obvious that there is an urgent need for new agents for the treatment of tuberculosis; however, the new potential compounds face some major hurdles before they become our stock compounds. The new guidelines are thorough, but are, of necessity, time-consuming. This time element is even more significant with trials which, by definition, must last at least 3.5–4 years for HIV-positive patients and 6–7 years for HIV-negative patients [183]. This leads to three key critical questions. (i) For how long must studies be performed? (ii) Who will pay for this research; is it to be the pharmaceutical companies, the health authorities or independent organisations? (iii) If these new agents are satisfactory, will they be affordable for the patient or the health systems of both developed and developing nations?

BCG: IMMUNISATION AGAINST TUBERCULOSIS?

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Tuberculosis has been a scourge of mankind for at least 6000 years. It has killed millions of people and millions of animals, principally cattle. Thus, it is not surprising that great efforts have been made to prevent tuberculosis by immunisation. Following the success of antitoxin therapy in diphtheria and tetanus, Maragliano and Marmorek separately prepared sera by inoculating horses with filtrates and extracts of tubercle bacilli and even streptococci from the sputum of patients with tuberculosis. Doses were given by injection or per rectum every other day for prolonged periods. The latter route was in an attempt to avoid serum sickness. There was no proof of efficacy and this therapy ceased [214].

The first live 'attenuated' vaccine was produced by Behring in 1902. Human tubercle bacilli were attenuated by drying or passage in glycerine broth. It was administered to calves but proved insufficiently attenuated. Nevertheless, it helped to establish the principle of using live attenuated organisms from one species in another species or vaccine. In 1903, Friedmann produced a vaccine isolated from the lungs of a tuberculous sea-turtle. The 'turtle-vaccine' was

ineffective and produced local abscesses. Subsequently others (Webb, Williams and Selter) suggested that immunisation should be performed with very low numbers (1–2 cfu) of live virulent bacilli followed by a gradual increase in numbers of bacilli over time. This also proved too dangerous. In 1923 Dreyer produced a defatted and dewaxed diaplyte vaccine. The expectation was that this would expose protective antigens. It too was dangerous and ineffective [214]. The only vaccine to stand the test of time has been BCG (Bacille Calmette-Guérin).

What is it?

The strain used in BCG was originally isolated from a cow by Nocard (Souche Lait Nocard). Leon Calmette was director of a research institute in Lille and in collaboration with Camille Guérin began a series of subcultures of the mycobacterium. They noticed that ox bile was effective in producing fine emulsions of tubercle bacilli and that the morphology and virulence of the bacterium were altered. In 1906 they began serial subcultures of their supposed strain of *M. bovis* on ox-bile glycerine-potato medium at *c.* 3-weekly intervals. After 231 subcultures (13 years later) they considered that it was sufficiently and permanently attenuated. It was shown to produce a mild febrile illness in calves but was eliminated without production of tubercles. It did not produce illness in guinea-pigs but was able to protect them against subsequent challenge by *M. tuberculosis* [214, 215]. Their persistence at this task deserves high praise since, for example, Calmette was arrested by the German forces occupying Lille during the 1914–18 war.

In 1921 a neonate born to a mother with tuberculosis was the first human vaccinee. The oral dose produced no side-effects. BCG was subsequently introduced as a vaccine for newborns in several countries but without performing controlled trials. In 1930 in Lubeck some 200 infants developed tuberculosis and 72 died following oral administration of BCG. A subsequent report showed that a virulent strain of *M. tuberculosis* which was kept in the same laboratory had contaminated BCG stocks and was responsible for the tragedy [214, 215].

In 1927 Wellgren introduced the intradermal route of administration of BCG and this was employed widely in Scandinavia. Carefully controlled trials were conducted in North American Indians from 1936 to 1956 which demonstrated efficacy.

The UK was one of the last countries to take up routine BCG vaccination, but careful MRC trials conducted on 50 000 school children from 1950 onwards demonstrated efficacy. The incidence of tuberculosis in the BCG vaccinated group was 0.4/1000 compared with 1.9/1000 in the unvaccinated. This represented a 79% decrease in disease by

vaccination [216] and there was still substantial protection 7–10 years after vaccination.

From 1948 onwards, under the auspices of WHO and UNICEF, BCG vaccination campaigns were begun throughout the world. It has been estimated that between 1948 and 1974, 1.5 billion persons had been vaccinated with BCG [215]. BCG has been included in the WHO expanded programme of immunisation and it is estimated that by the year 2000, 100 million doses of BCG will be required [215].

Production and quality control

BCG is an old vaccine, developed before our increasing understanding of biological variability. Unfortunately, seed stocks of the original BCG vaccine have not been kept. This has resulted in a variety of phenotypically and genotypically different strains of BCG being produced as vaccines throughout the world. Serial subculture has resulted in a plethora of strains of BCG with differing viability, immunogenicity, reactogenicity and residual virulence. Indeed, several different colonial variants have emerged which have reverted to 'normal' on further subculture. A recent study of 25 BCG isolates representing 16 reference strains produced from 11 to 15 different large restriction fragment patterns (genotypes) depending upon the restriction endonuclease used [217]. The patterns produced more closely resembled those of *M. tuberculosis* than *M. bovis*! Freeze-dried vaccine was introduced in 1956. This together with the use of seed-lot methodology should prevent further drift of vaccine strains. Nowadays *c.* 92% of BCG production is derived from three seed-lots (Glaxo-1077, Tokyo-172, or Pasteur-1173P2).

The method of culture of BCG may also affect its properties. Currently three methods are available [215]. These are: (i) aerobic culture on the surface of Sauton liquid medium; (ii) as submerged culture in Sauton or other liquid media containing detergents such as Tween 80 or Triton WR 1339; or (iii) in aerated and stirred bioreactors. The final method seems not to alter viability of immunogenicity too greatly and has the advantage of greater yields. This is of more than passing importance for production of the estimated 100 million doses needed by the year 2000.

Having produced BCG in sufficient quantities, it is necessary to provide a vaccine of uniform quality. BCG is subjected to various laboratory tests. Batches are tested for identification (by acid-fast stains), purity (no other contaminating micro-organisms) and safety (inoculation into guinea-pigs for absence of virulent mycobacteria). Potency has been expressed in terms of protein content or more recently by viable units (VU 10⁶ ml). Both of these are subject to considerable variation; for example, suspensions of BCG containing clumped bacilli will give lower viable counts on solid media, or inclusion of dead BCG in the vaccine will

give a higher protein content per viable bacterium. Vaccine lots are tested for thermostability as they must be transported and stored under varying temperatures throughout the world. Residual virulence is tested by inoculating defined VU 10^6 /ml into mice (looking for persistence in spleen) or into guinea-pig skin (looking for skin reactivity). Tuberculin allergy is tested by tuberculin challenge to guinea-pigs previously inoculated with a set dose of vaccine. Finally, immunogenicity is tested by immunising mice or guinea-pigs with set doses of BCG and subsequently challenging with virulent *M. tuberculosis* (H37Rv), and comparing survival time with that in unimmunised animals.

From the above it is clear that there is tremendous variability in BCG and this may affect efficacy. The other important variables are the mode of administration of vaccine and the age at immunisation. The original administration of BCG was by the oral route but this was abandoned because of variable absorption. The subcutaneous route gave rise to cold abscesses and this was replaced by intradermal inoculation. The multiple puncture method was introduced in 1937 but this may result in a low dose of BCG being delivered. The scarification method is supposedly more effective than multiple puncture with less local reaction. Recently, it has been suggested that the aerogenic route might induce better cellular immunity and protection [218].

Calmette originally gave BCG to neonates and neonatal administration is policy in many countries. In such groups, duration of immunity might be a problem. The UK MRC trials were conducted in school children [216]. They demonstrated 79% protection with immunity persisting for 10 years or more. Whether similar efficacy can be expected at all ages or on re-vaccination is unclear.

Adverse effects

In general BCG does not produce severe neurological or fatal sequelae [219]. However, it is associated with minor local adverse events. Almost all vaccinees experience induration and ulceration of the BCG site. BCG abscesses can occur and persist but may be due to *Staphylococcus aureus* or other pyogenic bacteria. Regional adenitis occurs at a frequency of 0.1–38/1000 vaccinees [220]. In a recent study in Jamaican children, 9.5/1000 developed axillary adenitis but the rate was greatest (19.2/1000) in those vaccinated in the first 6 weeks of life [221]. It is noteworthy that BCG was isolated from only 17% of these cases of axillary adenitis but *S. aureus* and gram-negative bacteria were found in 31% [221]. Osteitis develops in 0.01–330/10⁶ vaccinees [220]. Disseminated BCG infection was reported to occur at a rate of 4/100 000 neonatal vaccinees in Sweden during 1979–89 [222]. The rate is usually < 1/10⁶ vaccinees. In Chile, persistent or disseminated BCG infection occurred at a rate of

3.5×10^6 vaccinees [223]. However, each of the children had some form of immunodeficiency. This of course raises the newer question of BCG vaccination in HIV/AIDS. There are case reports of disseminated BCG infection in patients who were subsequently proved also to be infected with HIV [224, 225]. However, studies have demonstrated that HIV infection does not increase the risk of complications of BCG vaccination [226, 227]. Clearly BCG should not be given to patients with AIDS [228].

In addition to immunodeficiency, suggested reasons for adverse events include the strain of BCG, mode of inoculation (i.e., subcutaneously in error), potency of vaccine batch, previous tuberculin sensitivity and HLA haplotype [229].

Does it work?

A major problem in answering this question is the wide variation in BCG strains used, modes of administration, populations and age groups studied, duration of observation, case finding, study design and statistical evaluation [220, 230]. Early trials with liquid BCG in neonates or in British school children demonstrated reasonable efficacy [214, 216, 220, 230]. The first large carefully controlled trial with freeze-dried vaccine was undertaken by the Indian Medical Research Council in a population of c. 360 000 persons in 209 villages and one town west of Madras in south India [231]. Two BCG seed lots were used and two dosages (by protein concentration) were compared with placebo. All ages (except, surprisingly, neonates) were included following initial tuberculin testing and radiography (followed by sputum microscopy and culture if suspicious of tuberculosis) was offered to all those over 10 years old. Subject identification was by finger- or palm-print. Follow-up was at 2.5 year intervals and case ascertainment was by tuberculin testing (for subjects given placebo), X-ray and sputum microscopy and culture. The trial showed no protective effect of BCG vaccination, indeed, there was a slight excess of cases in the vaccinated group [231]. The efficacy of BCG in tuberculosis prevention in selected trials is shown in Table 12. Clearly there is tremendous variability in the results. Recently meta-analysis (Table 13) of seven randomised trials and 10 case-controlled studies of BCG has been reported [232]. Overall, this demonstrated between 49% and 63% protective effect (primarily against pulmonary tuberculosis). As might be expected, the protective effect was greatest against bacteraemic disease (64% protection from TB meningitis and 78% protection against disseminated disease) or death (71%). Some have expressed concern over the validity of averaging BCG trials [220]. However, Colditz *et al.* were exhaustive in their search for trials and stringent in their exclusion criteria. From the large 95% confidence intervals it is clear that there was a great variation between different trials. Analysis showed that 41% of variability was related to geographic

Table 12. BCG and tuberculosis prevention

Type of study	Age group	Number studied	Protection (95% confidence intervals)	Follow up (years)
Randomised trials				
Haiti	< 20 years	3174	79% (41–99)	3
Canada (Cree Indians)	> 3 months	609	80%	6.5
UK	14 years	26 465	77% (67–81)	20
USA (Indians)	0–19 years	2992	75% (68–81)	20
USA (Georgia & Alabama)	> 5 years	34 767	14% (–30–50)	14
South India (Madras)	> 1 month	351 853	21% (–20–20)	12.5
USA (Georgia)	6–17 years	4639	236% (–80–70)	20
Case-controlled trials				
Brazil (Sao Paulo)			89% (81–92)	
UK (Birmingham)			52% (28–78)	
Cameroon (Yaounde)			50% (30–60)	
Papua New Guinea			38% (2–60)	
Colombia (Cali)			2% (–40–48)	
Argentina (Santa Fe)			0 (–30–30)	
Contact studies				
UK (non-Asians)			60% (28–85)	
UK (Asians)			50% (10–80)	
Togo (Lome)			48% (30–60)	
UK (Edinburgh)			40% (10–60)	
Thailand (Bangkok)			36% (18–48)	

Adapted from refs. 220 and 230.

Table 13. Meta-analysis of BCG efficacy

Studies (number analysed)	Relative risk (95% confidence interval)	Protection (%)
Randomised trials (7)	0.37 (0.18–0.74)	63
Case-controlled trials (11)	0.50 (0.39–0.64)	50
Alternate (2) + systematic (4) allocation trials	0.49 (0.34–0.70)	51
Mortality as end-point (7)	0.29 (0.16–0.53)	71
Infant vaccination (8)		
TB meningitis as end-point (5)	0.36 (0.18–0.70)	64
Disseminated TB as end-point (3)	0.22 (0.12–0.42)	78

Adapted from Colditz *et al.* [232].

location. BCG showed greater efficacy the further away the population was from the equator. Data and case validation accounted for 30% of the variability. Interestingly, only 6% of the variability was related to the age of vaccinees and 4% to the date of the study. The BCG strain used had no effect on variability [232, 233].

Another way of examining the efficacy of BCG is to compare the incidence of tuberculosis in countries where BCG has been used and those where it has not. The rate of decline in tuberculosis in the UK and Scandinavia where BCG has been used is similar to that in the Netherlands where it was not [234]. This of course does not mean that BCG is ineffective but that factors other than artificially induced cell-mediated immunity contribute to decline in tuberculosis.

Criteria have been proposed for the discontinuation of BCG immunisation [235]. In Sweden, routine BCG immunisation of neonates ceased in 1975 except for those born to foreign parents [222, 236]. This was accompanied by a six-fold increase in tuberculosis in Swedish children (0.18 cases/10⁵ person years *versus* 1.28 cases/10⁵). A similar study in Czechoslovakia also showed an increase in numbers of cases of tuberculosis

[237] but concluded that the advantages and disadvantages of BCG immunisation were in balance [238]. By comparing tuberculous incidence pre- and post-BCG discontinuation, a protective efficacy of 80% was calculated [238]. Interestingly, the incidence of *M. avium-intracellulare* lymphadenitis was higher in the BCG-unvaccinated than the vaccinated group [239].

Where next?

From the foregoing it is clear that BCG is not an ideal vaccine. At best it provides 80% protection and the duration of immunity is variable. In comparison with more recent vaccines, such as that against *Haemophilus influenzae* (b) [240], BCG performs poorly. However, the incidence of tuberculosis is high in developing countries and is increasing after a long period of decline in developed countries. In addition, the emergence of multi-drug-resistant strains of *M. tuberculosis* is beginning to compromise the use of isoniazid chemoprophylaxis. BCG is the only tried and tested vaccine currently available. Improvements in its efficacy might be possible with optimal formulations, different modes of administration [218] and by understanding why vaccine failures occur. It has, for example, been suggested that BCG vaccination in individuals with pre-existing *M.*

tuberculosis infection but with weak tuberculin sensitivity might result in clinical tuberculosis and apparent vaccine failure [241]. In countries where there is a high incidence of tuberculosis, neonatal BCG administration would help to circumvent this problem. It is also conceivable that improved 'new' tuberculins might provide a more sensitive assessment of *M. tuberculosis* exposure [242]. *M. microti* ('the vole vaccine') proved as effective as BCG in the UK MRC trials [216] and should be investigated. It is thought that BCG has the potential to induce a destructive delayed-type hypersensitivity (TH2) rather than protective cell-mediated immunity (TH1) and that the chance of this varies between individuals. Whether *M. microti* or even *M. vaccae* induce the correct response is discussed in the next article. Finally, much work is needed to define protective epitopes on *M. tuberculosis*. This could eventually lead to effective recombinant vaccines or genetically attenuated *M. tuberculosis* vaccines [215, 220].

At present it is clear that BCG will continue to be used. Indeed, it is being used in immunisation against leprosy (*M. leprae*) and Buruli ulcer (*M. ulcerans*) and for immunostimulation in neoplasia [215, 243]. Clearly it is not ideal but at present it is all we have.

IMMUNOTHERAPY FOR TUBERCULOSIS WITH *M. VACCAE*

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Introduction

Robert Koch introduced the first scientifically based treatment for tuberculosis in 1890 [244]. This consisted of repeated injections of tuberculin into the skin, maximising the necrotising element of immunity. This could effectively 'cure' the patient, but in generalised disease or that involving organs that could not withstand additional destruction, the patients died. The tuberculin shock syndrome associated with an increase of circulating tumour necrosis factor (TNF) and sensitisation of the tissues to its effects could result in collapse and death from shock within a few hours of injection of the large doses of tuberculin. Koch's syndrome cured some patients and killed others, evoking the concept of a double-edged sword. Following Koch, many others modified his therapy with variable success.

Notably, Friedmann [245] used a suspension of live 'Schildkrotentuberkelbazillus', or *M. chelonae*, believing that the immunotherapeutic agent should be an environmental species capable of causing a limited lesion when injected into the skin, rather than a

derivative of *M. tuberculosis* itself. Friedmann's product, called Anningzochin, is still available on the market for use in a wide variety of conditions.

Spahlinger developed several treatments for tuberculosis based on his concept that the immunotherapeutic agent should be as similar as possible to the bacilli in the lesions. Thus he grew tubercle bacilli of human type on media enriched with human serum for treating human disease, and bovine tubercle bacilli on media enriched with bovine serum for disease in cattle. He wished to use bacilli killed gently, thus culture tubes were sealed after good growth had been obtained, and left for a year at room temperature in the dark, when they would no longer grow on subculture, and he assumed them to be dead. His organisms may well have been alive, but in stationary phase [246]. Spahlinger also realised that bacilli in the tissues are stressed, and for treatment of some forms of tuberculosis he heat-stressed bacilli during culture [247]. Such preparations were used directly as immunotherapeutic agents, or to raise antisera in the black Irish hunters (horses) that he favoured. Many patients were treated with injections of these preparations and, as with Friedmann's treatment, Spahlinger's therapies appear to have been successful in many seriously ill patients.

These early immunotherapies discovered the importance of secreted antigens (Koch), common mycobacterial antigens (Friedmann) and stress proteins (Spahlinger). Their major disadvantage was their lack of drugs to reduce the number of bacilli in the tissues, a situation only seen today in infections with multi-drug-resistant tubercle bacilli (MDR-TB).

In good conditions, 6 months of modern chemotherapy results in cure of >98% of patients, but largely because of costs, poor prescribing and patients defaulting, tuberculosis has thrived. The problem has been made worse by increasing susceptibility associated with HIV seropositivity, poor social conditions in big cities and the development of bacterial drug resistance. The problem would be much less if the length of treatment regimens could be significantly shortened without loss of efficacy. Tubercle bacilli can persist alive in the tissues in an almost non-metabolising form in which they cannot be killed effectively by drugs. Three-quarters of modern regimens are to provide drugs to kill the awakening persister. The chance of developing drugs that can kill bacilli in their resting form is small, yet the bacilli are antigenically recognisable and should be susceptible to immune destruction. The variable success of BCG vaccine and the ability of the majority of infected people to live with viable tubercle bacilli in their tissues without developing disease shows that immunity can be highly effective against tubercle bacilli [248]. If patients with clinical tuberculosis had this protective immunity returned after the early bactericidal action of drugs

had killed over 95% of bacilli, then they should be able to cope with the remaining bacilli without further treatment. It is the aim of immunotherapy to achieve this [249].

Development of M. vaccae

Amongst the environmental mycobacteria found to prime for success of BCG against leprosy in Uganda was a strain of *M. vaccae* from which a stable variant was derived that has provided the basis of our immunotherapeutic approach. Killed suspensions of this organism (NCTC 11659) are potent inducers of immune recognition of group i, common, mycobacterial antigens, which are extremely important in the induction of protective immunity to mycobacterial disease [249, 250]. This is illustrated by the efficacy of BCG against leprosy [251], lack of T-cell responses to these antigens in patients with tuberculosis [252] and the ability of any mycobacterial species to induce some protective immunity from others in experimental animals.

At first thought of as an additive to improve the efficacy of BCG, animal studies showed that *M. vaccae*, like *M. leprae*, is more immunogenic when dead than when alive. The combination of 10^7 killed *M. vaccae* plus 10^6 live BCG is more effective as a vaccine against leprosy in children living in close contact with the disease than is BCG alone [253, 254]. When used alone, 10^8 killed *M. vaccae* is about as effective as the combination with BCG.

The value of killed *M. vaccae* as an immunotherapeutic agent was suggested both by the enhanced recognition of group i, common, mycobacterial antigen found after vaccination of healthy persons, and by a series of mixed skin test studies [255]. Sonicates of *M. vaccae* and of a few other species, when mixed with reagents expected to induce large and potentially necrotic responses, controlled the size and quality of the response locally and at distant sites [256] where the reagent being controlled was injected alone. Might it not also exert control over what goes on around the bacilli in clinical lesions?

The first studies of M. vaccae in tuberculosis

Following demonstration by skin testing of improved recognition of common mycobacterial antigen in English tuberculosis patients given an injection of *M. vaccae* during the course of their chemotherapy [257], studies were carried out in Kuwait in patients randomised to receive *M. vaccae* or placebo 1 month after starting a full course of chemotherapy. Biochemical, haematological and immunological tests showed the optimum dose to achieve immune recognition of common mycobacterial antigens to be 10^9 bacilli, a result foreshadowed by studies on leprosy patients. There were no adverse side-effects, and *M. vaccae* killed by autoclaving was found to be more effective

than that killed by irradiation used in earlier studies [258].

Clinical evaluation of M. vaccae in tuberculosis

A blinded and randomised study of 10^9 irradiated *M. vaccae* given after 6 weeks of chemotherapy carried out in The Gambia was the first in which clinical evaluation was the major criterion. Associated with increased cure rate and survival, recruitment finished before sufficient patients had entered for statistical significance to be achieved except in subgroups. Subsequent studies with autoclaved *M. vaccae* given ≤ 1 month after starting chemotherapy for pulmonary tuberculosis, were carried out in Nigeria, Vietnam and Argentina, and all of these have shown important benefits for the patients. A small study in Rosario, Argentina, showed that all clinical parameters, and especially radiological changes ($p < 0.05$), were improved in those receiving *M. vaccae* [259]. Similar results were obtained in a pilot randomised and blinded study in Hanoi, Vietnam, in which patients received an injection of *M. vaccae* 1–2 weeks or 1–2 months after starting a 9-month course of chemotherapy. The overall cure rate a year after completion of chemotherapy was 36 of 44 for the placebo group and 39 of 41 for the immunotherapy group ($p = 0.057$).

In Kano, Nigeria, conditions were very different, and far from completing chemotherapy, drugs of doubtful quality had to be bought at high price by the patients resulting in inevitably high rates of non-compliance [260]. Other problems included patients presenting with advanced disease, and one-fifth of them being HIV seropositive. Under such disadvantages *M. vaccae* given 1–3 weeks after starting chemotherapy had a remarkably beneficial effect. Follow-up *c.* 1 year after diagnosis found that none of 34 receiving immunotherapy had died; they showed an increase in weight of almost 8 kg, a fall in ESR of 42 mm in 1 h, and 22 of 33 were sputum negative for acid-fast bacilli (AFB) by microscopy. In contrast, 19 of 47 receiving placebo had died; survivors had increased in bodyweight by 2 kg, ESR had fallen by 15 mm and only 4 of 26 were sputum negative for AFB ($p < 0.005$ for all these parameters).

Studies on tuberculosis in progress

A study is almost completed in Romania, and others are in progress in Argentina, Pakistan, Romania and Vietnam, and a phase III trial is recruiting in South Africa. Data are impressive from the initial study in Bucharest and Brasov in Romania, in which patients were randomised to receive *M. vaccae* or placebo after 1 month of a 6-month course of chemotherapy. Specially notable has been the rapid resolution of radiological signs, including closure of cavities, improved regain of weight and fall in ESR ($p < 0.01$ for each parameter). Although at the end of chemotherapy

for new cases of drug susceptible disease there was no significant difference in cure rate between the groups, results were better after immunotherapy in patients infected with drug-resistant bacilli and in those with chronic tuberculosis, both the most difficult types of disease to treat.

The new studies in Rosaria (Argentina) and Peshawar (Pakistan) are of patients paired on the basis of X-ray appearances to receive placebo or *M. vaccae*. The new study in Romania is of putting an injection of *M. vaccae* or of saline after 1 week of chemotherapy, into the National Tuberculosis Programme in six of the 43 health control areas of the country. At the moment two centres are recruiting and a third is about to start. Pilot studies of reducing the period of chemotherapy by the addition of an injection of *M. vaccae* from the current 9 months to 6 months and then 4 months are progressing in Hanoi, Vietnam. In Ho Chi Minh City, Vietnam, comparisons are under way between chemotherapy alone, chemotherapy plus a single injection of *M. vaccae* after 1 week, and chemotherapy with two injections of *M. vaccae* given after 1 week and 8 weeks.

A phase III trial in Durban, South Africa, has completed its pilot study and almost one-third of the 370 planned admissions have entered the main study. Expected to be completed in 1996, advanced analyses of safety data are expected. Patients will be followed up for at least 2 years after completion of chemotherapy.

M. vaccae in multi-drug-resistant tuberculosis (MDR-TB)

A study completed in Mashad, Iran, was devoted to this problem [261], studies in Romania and Vietnam have included patients infected with drug-resistant organisms, and a study is in progress on MDR cases in Calicut, India. In the Iranian study, there was a historical control group in which only one of the last 100 such patients had achieved bacteriological cure with the available drugs alone. By the addition of up to four injections of *M. vaccae* 11 of 42 patients were successfully cured ($p < 0.0001$) and remained sputum negative for AFB for at least 2 years. To summarise results available, immunotherapy with *M. vaccae* is about as effective in new patients, or at least those with a history of no more than 2 years of chemotherapy, as it is in patients infected with drug-sensitive bacilli. However, patients who have received chemotherapy irregularly over 3 years or more are much less responsive, and may require repeated injections of *M. vaccae* at 2-month intervals. The mean history of treatment for patients cured by a single dose of *M. vaccae* was 14.5 ± 2.6 months, and for those cured after three or four injections it was 81.1 ± 37.1 months ($p < 0.01$). So far up to six injections have achieved a cure rate between 20 and 30%. With such patients it is often

difficult to distinguish between the effects of immunotherapy and of concurrent chemotherapy in countries where newer drugs are available to which the bacilli are sensitive *in vitro*. In Vietnam, after failing two courses of chemotherapy patients are discharged on isoniazid monotherapy, and it is presumed that they all die. In this situation, up to six injections of *M. vaccae* at 2-month intervals have resulted in bacteriological cure in 15 (24%) of 62 patients. Currently, the number of injections is being increased to 10 in case additional patients can be cured.

HIV seropositive tuberculosis and M. vaccae

A proportion of the patients entered in each of our African studies have been seropositive for HIV-1 or -2. No special follow-up was carried out in The Gambia on the 11 HIV-2 seropositive patients, but in Kano, Nigeria, where 19% were seropositive for HIV-1, and a few were positive for HIV-2, follow-up was performed. All the patients started with severe tuberculosis, and often other AIDS-defining conditions. Of those receiving an injection of saline, six of nine died within a year, and at least eight of the nine were dead in 2 years. Of the eight who received *M. vaccae*, all were alive at follow-up after 2 years ($p < 0.0005$) [262].

About one-third of all patients entered in the phase III trial in Durban, South Africa, are seropositive for HIV-1 and should provide important data on the effects of *M. vaccae* in the dual infection. A small study is progressing in New Hampshire, USA, in which HIV seropositive children are receiving *M. vaccae* or hepatitis B vaccine as placebo, attempting to prevent them from developing *M. avium* infection.

Discussion

So far, much information has been obtained from our pilot studies about the use of *M. vaccae*. A preferred strain has been selected and deposited as NCTC 11659, a suitable dose (10^9 , equivalent to 1 mg wet weight) has been identified and organisms killed by heat have been found to be better than those killed by irradiation [258]. The reagent is given as an intradermal injection of 0.1 ml over a deltoid muscle. Its use in immunotherapy has been singularly free of side-effects. Local reaction is less than that of school children to BCG vaccination, and no more than 2% have reported mild fever or headache in the 12 h after injection. Repeated doses produce marginally reduced local reactions, and patients can be reassured about their likely reactions to subsequent doses.

The mode of action of *M. vaccae* remains elusive, but it is unlikely to be due to a single substance, or *via* a single pathway. The 'active' ingredients are presented with the powerful adjuvant of mycobacterial cell wall, which differs between different mycobacterial species.

That of tubercle bacilli promotes antibody production through enhancement of maturation of T helper cells type 2 (TH2). That of *M. vaccae* enhances cellular rather than humoral responsiveness through maturation of TH1 cells. The former is associated with establishment of infection and development of disease, whereas the latter promotes antibacterial, protective immunity. The mechanism underlying tissue destruction in the immunopathology of tuberculosis, resulting in caseous necrosis, resembles the Koch phenomenon and is associated with mixed TH1 and TH2 activity.

T helper cell maturation seems to be controlled by the balance of adrenal cortical hormones, themselves under hypothalamic control, and whatever controls that. *M. vaccae* might intervene at the adrenal, hypothalamic, or higher centres. Possibly it blocks the effect of TNF-enhancing activity of tubercle bacilli (TEA) on ACTH, although such an action is too specific to tuberculosis and would not explain the action of *M. vaccae* in other conditions. Whatever the mechanism, the ratio of levels of circulating cortisol and dehydroepiandrosterone (DHEA) and their metabolites controls T-cell maturation. Increased cortisol promotes TH2, and increased DHEA promotes TH1. Levels of DHEA and its metabolites are significantly reduced in untreated tuberculosis and in HIV seropositivity.

M. vaccae re-instates cellular immune responsiveness to group i, common, mycobacterial antigens, recognition of which is a key part of antibacterial immunity [249,250]. Although *M. vaccae* is rich in these antigens, we cannot tell what part they play in its action, since responses to them seem always to be TH1, and their recognition may be a marker of such activity. Cellular responsiveness to common mycobacterial antigens is significantly reduced in rheumatoid arthritis [263], HIV seropositivity [264] and the silent, seropositive, stage of *Trypanosoma cruzi* infection [265]. Significantly, group i, common mycobacterial antigens include the mycobacterial stress proteins, which share major sequence homology with their human tissue counterparts. Antibodies to stress proteins are associated with TH2 predominance. They often bind to both mycobacterial and human stress proteins. Such autoantibodies have been demonstrated in rheumatoid arthritis, schizophrenia and other auto-immune conditions which may be associated with infection. We do not know whether the beneficial effects of *M. vaccae* found in such conditions are due to preferential presentation of stress proteins or other shared antigens, or to the down-regulation of TH2.

The beneficial effects of *M. vaccae* can be extremely long-lasting; for example, the vaccination of close contacts of leprosy patients can readily be detected 8–10 years later by an enhanced responsiveness to skin tests with Leprosin A [253], and immunotherapy of leprosy patients with *M. vaccae* improves blood flow to the fingertips for at least 18 months [266]. Long

projected effects may be due to continuous boosting by environmental mycobacteria, but in MDR-TB of the chronic type, repeated injections at 2-month intervals are required, suggesting that tubercle bacilli themselves may not boost beneficial effects. However, it has been suggested that the beneficial effects of *M. vaccae* on HIV seropositive tuberculosis patients might be due to boosting by tubercle bacilli.

Mycobacteria are generally thought of as intracellular pathogens and in its earliest phases this is true of tuberculosis. As disease progresses into caseous necrosis and cavity formation, more and more bacilli become extracellular. In chronic tuberculosis the patient achieves a balance with his disease and may live for years shedding millions of tubercle bacilli with the disease extending very slowly. Under these conditions the great majority, if not all, of the bacilli are extracellular in dead tissue lining cavities or on the surfaces of air passages denuded of epithelium. Isoniazid and rifampicin are very effective against extracellular bacilli, but this effect is lost with the development of multi-drug resistance. The condition of chronic open tuberculosis, well-known in the days before chemotherapy, is now seen in patients infected with MDR-TB. *M. vaccae* is thought to work by optimal activation of macrophages by TH1 lymphocytes to kill tubercle bacilli, which can be effective only when bacilli are intracellular.

Weight loss, weakness and intermittent fevers of chronic tuberculosis result from toxicity of circulating TNF enhanced by TEA absorbed from extracellular bacilli. These effects may be reversed by immunotherapy with *M. vaccae* without patients becoming sputum negative for AFB. First noted in Iran, early clinical improvement may not be associated with bacteriological cure in all cases. The effect wears off and does not usually follow the successive injections of *M. vaccae* required to achieve bacteriological cure. Repeated injections may achieve success by stopping further necrosis of tissue in contact with tubercle bacilli, eventually bringing the bacilli into contact with effective immune cells that can destroy them.

Conclusions

Autoclaved *M. vaccae* NCTC 11659 is very effective in the treatment of newly diagnosed pulmonary tuberculosis. The effect is independent of the drug resistance of the bacilli, and in preliminary studies appear to be equally effective in HIV seropositive and negative patients. Clinical symptoms improve faster and bacilli disappear from sputum more quickly, thus patients feel better faster, and may put fewer people at risk of contracting tuberculosis from them. This is particularly important in cases of multi-drug resistance. Minimal side-effects and the potential cheapness and simplicity of the reagent could make it rapidly available for worldwide use. If the promise shown in the initial

studies is borne out in larger trials, immunotherapy with *M. vaccae* will be a major factor enabling control of tuberculosis early in the next century.

Concluding comments

The conference emphasised the problems of individual case management and addressed novel approaches to diagnosis, chemotherapy and immunoprophylaxis. The potential of these new approaches is encouraging, but they are expensive and are unlikely to be applied routinely in the developing world. It is crucially important that appropriate, affordable and sustainable methods of tuberculosis control are supported in resource-poor countries. These objectives and their implementation have already been stated by both the WHO and the International Union against Tuberculosis and Lung Disease [267–270].

Within developing countries, the key to success remains the provision of support for adequate case finding and treatment of infective patients, and the integration of these services with other control programmes including those relating to HIV [271]. Control measures advocated in some Western nations, such as tuberculin screening programmes followed by isoniazid monotherapy for positive reactors [272] are usually inappropriate for developing nations, for which different strategies are needed [273, 274]. Simple measures to maintain the quality and increase the yield of sputum examinations in the laboratory [275] and in the clinic [276] deserve more exploration. The belated recognition of the risks of nosocomial transmission of tuberculosis, possibly multiresistant, coupled with the overburdening of hospital resources due to increased cases of HIV-tuberculosis co-infection, require a reappraisal of treatment programmes that incorporate a prolonged intensive initial phase of inpatient treatment [277]. Expensive patient isolation procedures and environmental control measures suitable in the USA [278, 279] are not the answer; instead there is an urgent need for more operational research that facilitates the provision of community-based therapy [280].

The difficulties of motivating administrators and medical staff to deliver 'model' care programmes in India have been graphically described [281, 282], and are compounded by the realities of trying to maintain the compliance of patients and the education of physicians outside model programmes to diagnose and manage cases appropriately [283, 284]. Co-infection with tuberculosis and HIV is already rising in the Indian subcontinent [285], and the potential dual epidemic in Asia is likely to be far worse than that seen in Africa. Some encouraging results have been reported from Nepal, where community-based case finding and treatment have been successful, despite the logistic problems of health-care delivery in remote

areas [286]. We can only hope that such success can be extrapolated to neighbouring countries and programmes.

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