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5 Immunology of mycobacterial disease

The clinical and histological features of mycobacterial disease are more the result of the immune reactions of the host than of the invasive powers of the pathogens. In the case of leprosy, in particular, these features enable a very accurate assessment of the nature of the patient's immune response to be made. The subject of mycobacterial immunology has become a very extensive one in recent years. The reader is referred to Chapters 25 to 29 in Bloom (1994) for detailed reviews of basic principles and to Fine (1994) for a thought-provoking and fascinating account of the relevance of immunology to disease control. The purpose of this chapter is to describe those areas of immunology that are relevant to an understanding of the pathogenesis of mycobacterial disease, the nature and significance of tuberculin reactivity, the principles of vaccination, the development of immunotherapy and the increasingly important interactions between mycobacteria and the human immunodeficiency virus (HIV).

Mycobacteria, in common with other intracellular parasites, owe their virulence to their ability to survive within macrophages. Protective immune reactions in mycobacterial disease are of the so-called cell-mediated type. Humoral immune responses, i.e. antibody production, certainly occur and many attempts have been made to utilize these for diagnostic purposes; but they do not appear to play a major role in host defence. There are two main types of protective cell-mediated immune responses. One type, which until recently was regarded as being the sole protective mechanism in mycobacterial disease, serves to enhance the ability of the macrophage to inhibit or destroy the invaders. The second type, now known to be of crucial importance to protection, is the ability of certain cells of the immune system to detect macrophages and other cells that are not controlling intracellular bacillary replication and to lyse them. This releases mycobacteria, thus enabling them to be engulfed by immunologically effective phagocytes. It has also been shown that the products of cell lysis inhibit mycobacterial growth *in vitro* and it is thus highly likely that such cell destruction *in vivo* generates an environment that is highly unfavourable to mycobacterial growth and survival (Dannenberg, 1993). This shift of emphasis is an important one as cell destruction was previously thought to be associated only with immunopathology and progression of disease.

Immune phenomena in mycobacterial disease – the basic principles

The immunological phenomena seen in mycobacterial disease consist, as in other infections, of recognition, response and reaction. In the first of these steps, the invading mycobacteria are recognized as being 'foreign'. In the second step the necessary defence mechanisms are alerted and recruited, while in the third the actual struggle between the mycobacterium and the host takes place.

Antigen recognition

Antigen recognition is a property of the lymphocytes, which are divided into two main sets: the B-cells (bursa- or bursa-equivalent-dependent) and the T-cells (thymus-dependent). The former mature into antibody-secreting plasma cells, while the latter are involved in the initial recognition of antigen, the regulation of the immune response by secretion of hormone-like chemical messengers called cytokines and in cell-mediated cytotoxicity. The T-cells are divisible into subsets according to their functions and by the presence of certain markers; namely, the cluster differentiation (CD) antigens detectable by means of monoclonal antibodies. The principal T-cell subsets are those bearing either the CD4 or CD8 markers. The former include the helper and inducer cells while the latter includes suppressor and cytotoxic cells. The association of CD antigens and functional activity is, in fact, not so clear-cut as originally thought as cytotoxic CD4+ cells have been described (Ottenhoff *et al.*, 1988; Flynn *et al.*, 1992; Lorgat *et al.*, 1992).

The activities of the different subsets of T-cells are largely due to the cytokines that they secrete. A number of well-characterized cytokines produced by cells of the immune system are termed interleukins. For a detailed review of these and related cytokines, see Hamblin (1993). It has been shown that CD4+ T-helper (TH) cells mature along separate pathways, resulting in cell populations with quite different functions (Mosmann and Moore, 1991). These are recognized by the nature of the cytokines that they secrete (Table 5.1) and are termed TH1 and TH2. There is also evidence that CD8+ (suppressor/cytotoxic) T-cells likewise mature along these pathways so the terms Type 1 and Type 2 are often used instead of TH1 and TH2. This division into

Table 5.1 Cytokines produced by TH1 and TH2 T-cells

Cytokine	*TH1*	*TH2*
Gamma interferon	+	–
Interleukin-2	+	–
Interleukin-4	–	+
Interleukin-5	–	+
Interleukin-6	–	+
Interleukin-10	–	+

two maturation types is of great relevance to the nature of the immune response following infection by a mycobacterium, as described below.

To complicate the matter further, helper T-cells are also divisible according to differences in the structure of the receptor molecules that bind to antigen. These receptors consist of two protein chains which, in most cells, are of types termed alpha and beta but a minority of cells have gamma and delta chains. The latter, termed gamma-delta (γ-δ) cells are usually CD4–/CD8– but a minority are CD8+.

Each lymphocyte bears a receptor that binds to just one of the thousands of possible antigenic determinants or epitopes. Accordingly, there must be many thousands of subpopulations of lymphocytes, each specific for just one epitope. An important stage of the immune response is that of clonal expansion, in which small numbers of antigen-specific lymphocytes proliferate to form clones of cells of sufficient numbers to mediate an effective immune response.

Before an antigen is able to induce such clonal proliferation it must be presented to the lymphocytes in a special way. This is the task of the antigen presenting cells (APCs) which include cells of the monocyte/macrophage series, dendritic cells of lymph nodes and scattered lymphoid tissues and the Langerhans cells of the dermis. Other cells may, under certain circumstances, also serve as APCs. The process of antigen presentation is shown in Fig. 5.1. The APCs engulf mycobacteria which contain a multitude of epitopes. Some of these epitopes are actively secreted (see Chapter 2) and some are somatic, and only released by bacteria that are digested, to a varying extent, by enzymes within lysosomes.

Mycobacterial epitopes are presented on the surface of the APC in close relation to host molecules which are products of the major histocompatibility

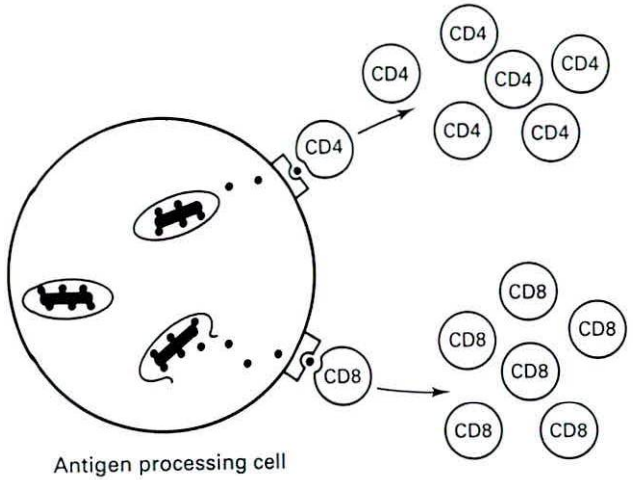

Fig. 5.1 Antigen presentation. A complex antigen is phagocytosed and degraded by the antigen processing cell (APC). Individual epitopes derived from antigens within phagosones are presented on the cell membrane by the Class II MHC (HLA-D) molecules to antigen-specific CD4 T-cells while those derived from antigen in the cytoplasm are presented by the Class I (HLA-A and HLA-B) molecules to CD8 T-cells (T). In each case, this process activates the T-cells which then proliferate to form clones

complex (MHC). There are two types of such molecules: Class I, coded for by the HLA-A and HLA-B genes, and Class II, coded for by HLA-D genes. The Class II molecules are principally found on cells with specific antigen presenting functions and activate the CD4+ helper/inducer T-cells. Class I molecules are present on all cells and activate the CD8+ suppressor/cytotoxic T-cells. Binding of antigen-specific lymphocytes to the epitope/HLA complex causes a signal to be delivered by the APC to the lymphocyte, inducing the latter to secrete interleukin-2 (IL-2) which mediates lymphocyte division and clonal expansion. Epitopes from pathogens within phagosomes are presented by MHC Class II molecules while those from pathogens that lie freely in the cytoplasm of the cell are presented by the Class I molecules. This difference in presentation enables the immune system to 'decide' whether to enhance the microbicidal power of the cell or to destroy it and thereby liberate its contents.

The immune response: macrophage activation

The next cells to enter the scenario are the macrophages which belong to the same cell lineage as the blood-borne monocytes. Macrophages are phagocytic cells but, unlike the other major class of phagocytes, the polymorphonuclear leukocytes, they are long-lived cells which settle in a given tissue or organ and adapt to the local environment. Thus, alveolar macrophages, osteoclasts, Kupffer cells of the liver and Schwann cells of the nerves are all specialized macrophages. Further, macrophages do not express their full antimicrobial potential unless they are 'activated'. Such activation is mediated by gamma interferon (IFN-γ) which is secreted by the clonally expanded population of CD4+ helper T-cells (Rook *et al.*, 1986).

In man, there is an additional step in macrophage activation involving vitamin D. Macrophages activated by IFN-γ produce a hydroxylase enzyme which converts inactive vitamin D into its active metabolite calcitriol which further activates the macrophage (Fig. 5.2; Rook, 1986). This may well explain the success of vitamin D therapy in the treatment of lupus vulgaris in the pre-chemotherapy era.

The activated macrophage differs from its resting counterpart in several respects. The cell membrane is much more motile – a phenomenon termed membrane ruffling. Random migration, glass adherence and the ability of the cell to phagocytose and kill micro-organisms are all increased.

In addition to its microbicidal activity, the macrophage synthesizes and secretes many important compounds that affect the pathogenesis of mycobacterial disease. These include some of the acute-phase reactant proteins, vasoactive peptides, and proteases that liquefy necrotic tissue and thereby contribute towards the formation of the tuberculosis cavity. In addition, macrophages produce a cytokine termed tumour necrosis factor (TNF-α; Flesch and Kaufmann, 1990). This cytokine has a protective function and treatment of mice infected with Bacille Calmette–Guérin (BCG) or *Mycobacterium tuberculosis* with antibody to TNF-α leads to rapid progression of disease (Rook and Bloom, 1994). On the other hand, TNF-α is an important mediator of tissue-necrotizing immunopathology as described on page 89. Tumour necrosis factor-alpha is also known as cachectin and its

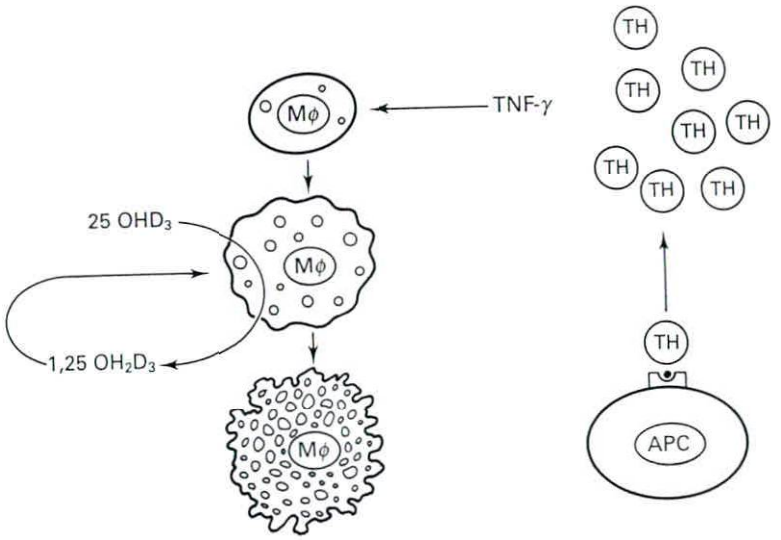

Fig. 5.2 Macrophage (Mϕ) activation. In response to antigen presented by the APC and subsequent clonal expansion of the T-helper cells, gamma interferon (IFN-γ) is released and activates the macrophage. This induces a hydrolase which converts vitamin D3 ($25\text{-}OHD_3$) into the more active $1,25\text{-}OH_2D_3$ metabolite which causes further macrophage activation. Data from Rook (1986)

systemic release is responsible for the extreme wasting associated with advanced tuberculosis (Beutler *et al.*, 1985).

An important characteristic of chronic infections is the formation of the granuloma which is a compact cordon-like aggregate, many cells thick, of activated macrophages around the site of infection. Macrophages in granulomas are termed epithelioid cells, from their morphological similarity to epithelial cells (Fig. 5.3). Many cytokines, including TNF-α, are involved in granuloma development and regulation. The granulomas were termed *tubercles* by Hippocrates who likened them to miniature tubers of plants; hence the name tuberculosis.

The immune reaction: events within the macrophage

In order to survive within a macrophage, a bacterium must be able to resist destruction by the wide range of non-oxygen-dependent and oxygen-dependent killing mechanisms of the infected cell. The former mechanisms include enzymes such as lysozyme, lipases and phosphatases and the latter include generation of reactive oxygen intermediates such as superoxide radicals, hydrogen peroxide and hypochlorite ions. In the mouse, nitric oxide and related reactive nitrogen intermediates (RNIs) generated from L-arginine provide powerful means of killing mycobacteria (Chan *et al.*, 1992) but it is not certain whether human macrophages generate RNIs (Rook and Bloom, 1994).

Fig. 5.3 A tuberculous granuloma showing whorls of epithelioid cells, some giant cells and central necrosis

The process of phagocytosis is shown in Fig. 5.4. An engulfed bacterium lies within a vesicle formed by invagination of the surface membrane. This vesicle, the phagosome, then fuses with the lysosomes which contain the bactericidal agents referred to above. There are three main strategies by which bacteria or other pathogens survive within the phagosome. First, phagosome/lysosome fusion may be inhibited. Secondly, the pathogen may cover itself with a protective layer that absorbs or neutralizes the bactericidal agents or, thirdly, it may escape from the vesicle and lie freely in the cytoplasm of the cell.

The extent to which mycobacteria use these strategies is controversial. *Mycobacterium tuberculosis*, in common with the protozoal parasite *Toxoplasma gondii*, inhibits phagosome–lysosome fusion but neither the mechanism nor the significance of this activity to intracellular survival are clearly understood (Draper, 1981). Mycobacterial pathogens also appear to survive the effects of exposure to reactive oxygen intermediates (ROI) on

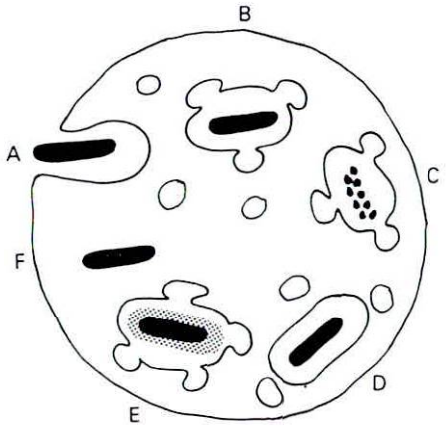

Fig. 5.4 Phagocytosis. A: Bacilli are engulfed by the cell membrane. B: The bacilli lie in a membrane vesicle – the phagosome – which fuses with lysosomes containing bactericidal substances. C: These substances destroy the bacilli. Mycobacteria avoid such destruction by – D: inhibiting phagosome/lysosome fusion; E: a thick capsule-like outer protective layer; and F: escape from the phagosome to lie freely within the cytoplasm

account of the thick outer layer of mycosides which, on electron microscopy, appears as an electron transparent zone surrounding the bacilli. Lipo-arabinomannan (LAM), which is present in all mycobacteria, and phenolic-glycolipid-I (PGL-1) in *M. leprae* also protect against ROI (Chan *et al.*, 1991). In addition, mycobacteria secrete the enzyme superoxide dismutase (SOD) which also protects against ROI (see Chapter 2, page 15).

Mycobacteria are able to escape from the phagosome and replicate in the cytoplasm (McDonough *et al.*, 1993). This appears to occur when the cell becomes immunologically effete and unable to control intracellular growth or when mycobacteria enter cells other than macrophages. Epitopes from the cytoplasmic location are presented to T-cells by the MHC Class I molecules which, as mentioned above, are present on all cells and facilitate cell lysis by CD4+ and CD8+ cytotoxic T-cells.

The early immunological events after infection

The events summarized above underlie the pathogenesis of primary pulmonary tuberculosis. The infectious particle is a small droplet of cough spray, about 5 μm in diameter, containing a few tubercle bacilli. After inhalation, this lodges in an alveolus, usually near the periphery of the lung, and the bacilli are engulfed by alveolar macrophages. If the macrophage cannot control bacillary growth, it is killed with release of the bacilli which initiate an inflammatory response which, in turn, attracts blood-borne phagocytes and other white cells, including natural killer cells and γ-δ T-cells. The latter cells recognize certain, as yet not clearly defined, mycobacterial components and cause the formation of a low-turnover granuloma of the 'foreign body' type. This to some extent, limits the progression of the disease process before the

development of the specific immunity mediated by CD4+ T-cells. There is evidence that early recognition of mycobacteria by γ-δ cells is required for subsequent activation of antigen-specific α-β CD4+ T-cells (Kaufmann *et al.*, 1993). Mycobacterial adjuvants (see page 105) induce granuloma formation and there is evidence that this may occur by a T-cell-independent pathway (Bancroft *et al.*, 1991).

Some tubercle bacilli in the primary focus are transported, probably within phagocytic cells, to the local lymph nodes, where antigen is processed and presented to antigen-specific lymphocytes as described above. Some bacilli are carried further afield in the lymphatics and blood stream and are responsible for the serious non-pulmonary manifestations of primary tuberculosis described in Chapter 8.

When the specific immune response has developed, the low cellular turnover 'foreign body' type granuloma gives way to the high turnover granuloma of immunogenic origin. In tuberculosis the granuloma consists of a central area of cheese-like necrosis, or caseation, surrounded by epithelioid cells and with lymphocytes in the outer zone. Some epithelioid cells fuse to form multinucleate cells (Langhans' giant cells). In many cases this protective immune response is sufficient to arrest the disease and destroy most of the mycobacteria. Collagen is then laid down by fibroblasts and the foci heal by scarring.

Similar resolution of early disease also occurs in many cases of leprosy and other mycobacterial infections, although it has not been possible to examine the sequence of events in the same detail as in pulmonary tuberculosis.

Protective cell-mediated reactions as described above are not the only immune phenomena associated with mycobacterial disease. Others, such as immunosuppression and delayed hypersensitivity, also occur and make the subject much more complex. Indeed these other reactions are of great relevance to the pathogenesis of the diseases.

Delayed hypersensitivity and the Koch phenomenon

A hypersensitivity reaction is defined as one which causes tissue damage. Four main types are recognized; namely, anaphylactic (Type I), antibody-dependent cytotoxic (Type II), immune complex-mediated (Type III), and delayed (Type IV). The first three involve antibody but delayed hypersensitivity is 'cell-mediated'. The tuberculin reaction is often cited as the classical example of the delayed type hypersensitivity (DTH) or Type IV reaction but, in fact, there are a number of different DTH reactions, some of which cause tissue necrosis and others which do not. Both types occur in mycobacterial disease and appear to bear quite different relations to protective cell-mediated immune reactions. For this reason, the relation between DTH and CMI has long been the subject of controversy.

In order to understand DTH it is necessary to look back to the original studies of Koch (1891) which were carried out in an attempt to discover a cure for tuberculosis. Koch inoculated tubercle bacilli into the flanks of

guinea-pigs and observed the ensuing events. After a week or two a small, firm nodule developed at the inoculation site and subsequently ulcerated. Viable tubercle bacilli were isolated from the ulcer which remained open until the animal died. About a month after inoculation local lymph nodes were enlarged and disseminated disease developed, leading to death three or four months later. When Koch gave a similar inoculation of bacilli into the opposite flank one month after the original infecting dose, a quite different lesion developed. After a day or two the skin at the second inoculation site became black and necrotic and then sloughed off leaving a shallow ulcer from which no bacilli could be isolated and which soon healed. Koch then found that he could elicit a similar reaction, subsequently termed the Koch phenomenon, by injecting either killed tubercle bacilli or a heat-concentrated filtrate of the medium in which the bacilli had been grown, a preparation termed Old Tuberculin.

The important point to note is that, although this reaction clearly led to the elimination of bacilli inoculated into the skin, the animals were nevertheless dying of systemic tuberculosis. This implies that either the reaction does not occur in the presence of tubercle bacilli in the deep tissues or, if it does, it is either non-protective or positively harmful. In fact, Koch himself put the matter to the test by administering Old Tuberculin systemically to patients with tuberculosis. Although there were a few remarkable cures in patients with disease of the skin or larynx there was little or no effect in those with deep lesions. Indeed, therapy in some patients led to a worsening of pulmonary tuberculosis and some developed 'tuberculin shock' which in a few cases proved fatal. Thus it appears that if the necrotic Koch phenomenon occurs on the surface, the bacilli-laden tissue easily sloughs off. If, on the other hand, it occurs in the lung or other internal organ, the bacilli and necrotic tissue remain *in situ*.

Koch's work on tuberculin would probably have been forgotten had it not been for the extensive studies of the Austrian physician Clemens von Pirquet who showed that dermal reactivity to a small quantity of tuberculin was indicative of past infection by the tubercle bacillus and thus of great epidemiological value (von Pirquet, 1907).

Post-primary tuberculosis and the Koch phenomenon

Conversion to tuberculin positivity in humans occurs about six to eight weeks after the initial infection. By then the primary lesion is, in many cases, well contained. Post-primary disease occurs months, years or even decades later and is the result of either reactivation of old, latent, foci of disease or of exogenous reinfection (see Chapter 8).

Post-primary lesions which, for unknown reasons, often occur in the apical regions of the lungs, are characterized by extensive tissue necrosis. This was observed by Sylvius in 1680 who wrote 'I may clearly communicate that I saw on many occasions glandulous tubercles in the lungs which sometimes contained various forms of pus as a section showed' (Sylvius 1680).

The extensive necrosis results in large, caseating, tumour-like lesions termed tuberculomas being formed. As in primary lesions, these lesions are acidic and anoxic, and they contain tissue-derived free fatty acids. Thus the number of viable tubercle bacilli within them is low.

The caseous material in the post-primary lesions is softened or liquefied by proteases liberated by macrophages and, if the lesion erodes into a bronchus, the softened contents are coughed out leaving a cavity. In distinct contrast to the closed lesion, the cavity is well-oxygenated and becomes an ideal breeding ground for tubercle bacilli. Thus the cavity wall contains millions of freely replicating bacilli which are behaving more like saprophytes than primary pathogens. In addition, large numbers of bacilli enter the sputum, rendering the patient open or infectious. Some cavities close spontaneously due to contraction of fibrous scar tissue, the anoxic conditions return and the number of bacilli decrease. In the pre-chemotherapy era, closure of cavities was achieved therapeutically by inducing lung collapse by artificial pneumothorax or by surgical resection of parts of the chest wall – an operation known as thoracoplasty.

It is therefore evident that immunological reactivity of the tissue necrotizing type has a profound effect on the pathogenesis of the disease and is an important factor in determining infectivity.

Tubercle bacilli escaping from cavities may cause secondary lesions in the lower lobes of the lung, in the upper respiratory tract and, if swallowed, in the alimentary tract (Chapter 8, page 166). On the other hand, lymphatic and haematogenous dissemination of disease is, in contrast to primary tuberculosis, uncommon. This is probably due to necrosis of the draining lymphatics and capillaries by the DTH reaction. An important component of the protection afforded by BCG vaccine is the prevention of serious forms of primary tuberculosis that result from haematogenous dissemination, and this may be due to the induction of DTH (Ladefoged *et al.*, 1976).

Protective immunity and immunopathology in tuberculosis

For many years the relationship between protective cell-mediated immunity (CMI) and DTH was the subject of much controversy. Some workers claimed that these are essentially similar phenomena but differ in degree while others argued that they are separate and distinct reactions.

During the two decades following the introduction of the tuberculin test as a diagnostic and epidemiological tool by von Pirquet, it was widely asserted that tuberculin reactivity was a sign and measure of immunity. This view was seriously challenged by Rich and McCordock (1929) and many other workers in the ensuing decades (reviewed by Bothamley and Grange, 1991). There is now considerable evidence that small tuberculin reactions correlate with protection but that larger ones are indicative of tissue-destroying hypersensitivity (Fine, 1994). While some have argued that the difference between a protective CMI reaction and a necrotic DTH reaction is one of degree, evidence that the differences are qualitative rather than merely quantitative has steadily accumulated.

A major step forward in the resolution of this controversy was taken by Rook and Stanford (1979). In a detailed study of mice experimentally infected with various mycobacteria, these workers observed that a reaction to tuberculin peaking at about 20 hours after skin testing appeared around 10 days after infection, while a reaction peaking at about 40 hours appeared a month or so after infection. The former reaction, which was non-necrotizing, resembled that demonstrable in mice infected with *Listeria monocytogenes* while the latter appeared to be the murine equivalent of the necrotic Koch phenomenon in guinea-pigs. These reactions were therefore termed the Listeria-type and the Koch-type reactions, respectively. Interestingly, the less mouse-virulent daughter strains of BCG and certain environmental mycobacteria not known to cause disease preferentially elicited the non-necrotic (Listeria-type) reaction, while the more virulent of the BCG strains and other mycobacteria that are pathogenic in the mouse elicited the Koch-type reaction. Furthermore, the induction of one type of reaction appeared to block the subsequent induction of the other. Thus a predisposition to respond to subsequent mycobacterial challenges with the 'Listeria-type' response could be induced by incorporating a rapidly growing non-pathogen, *M. vaccae* in the animals' drinking water. These findings support the concept that immune reactivity in man is determined by the nature of the immunologically effective contact with mycobacteria early in life – a phenomenon termed 'original mycobacterial sin' by Abrahams (1970).

The practical relevance of these findings came from the observation that the efficacy of BCG vaccination varies greatly from region to region (see page 97). One of the most plausible of many explanations of this variation is that the efficacy of this vaccine is predetermined by previous exposure to mycobacteria in the environment. In studies involving guinea-pigs, Palmer and Long (1969) found that such exposure afforded some protection against infection by *M. tuberculosis*. Vaccination with BCG increased this immunity but never to a level above that induced by BCG alone. It was therefore concluded that the full measurable effect of BCG is seen in populations not previously immunized by contact with environmental mycobacteria, but that elsewhere the observed effect is less as the population already has some naturally acquired immunity.

It could therefore be postulated that in regions where BCG is ineffective the population would have received sufficient 'natural vaccination' to induce maximum immunity before vaccination. Indeed, in this situation, BCG could even push vaccinated persons from a protective to a hypersensitive state, thereby reducing their resistance to disease. If this theory is correct, the vaccine should confer protection if given to neonates or young children before they experience a significant exposure to mycobacteria in the environment.

An alternative explanation of the apparent differences in the efficacy of BCG was advanced by Stanford and his colleagues on the basis of their description of the non-necrotizing and necrotizing tuberculin reactions described above and the finding that some forms of immunologically effective contact with mycobacteria induce the former reaction while others induce the latter (Stanford *et al.*, 1981b). While BCG induces a non-necrotic, protective, response in those not previously sensitized by mycobacteria, in others it boosts whatever pattern of reactivity has been 'imprinted' by environmental

contact. Thus if BCG induces or boosts protective immunity it will appear effective but if it boosts predetermined necrotic Koch-type reactivity it will not appear protective.

An observation of fundamental importance in clarifying the relation between CMI and DTH was the demonstration that the two reactions could be adoptively transferred separately in mice by different T-cell clones (Orme and Collins, 1984). Subsequently a lot of light was shed on the nature of this dissociation at the cellular level and on the way in which environmental mycobacteria predetermine subsequent immune reactivity by the demonstration of the two T-cell maturation pathways, TH1 and TH2 (see above, page 79). Bretscher has shown that contact with mycobacterial antigen, even if in too small an amount to induce detectable immune responses, may 'imprint' the immune system with a tendency to respond to subsequent contact with mycobacteria with either a TH1- or TH2-mediated reaction (Bretscher, 1992). Thus, though containing numerous different epitopes, a complex antigen such as a mycobacterium induces a remarkably unified pattern of immune responses; a phenomenon which Bretscher has termed 'coherence'.

The balance between TH1 and TH2 achieved by immunization, and thus probably by natural infection, is determined by a balance between various steroid hormones (Daynes *et al.*, 1991; Rook *et al.*, 1993, 1994). Glucocorticoids such as cortisol promote TH2 maturation while dehydroepiandrosterone (DHEA) opposes this effect and promotes TH1 maturation. An increase in cortisol levels relative to DHEA occurs in tuberculosis and AIDS and raises the possibility of rectifying this imbalance by an immunotherapeutic agent with TH1 adjuvant properties (Rook *et al.*, 1994).

Cytotoxic cells and protective immunity

Lysis of immunologically effete macrophages and other cells containing pathogens provides a means of exposing these organisms to effective immune mechanisms (Fig. 5.5). As mentioned above, there is now evidence that cytotoxic cells recognizing mycobacterial antigen are essential for protective immunity (Boom *et al.*, 1991; Flynn *et al.*, 1992). There are also less specific mechanisms for lysing infected cells, including natural killer cells, cytotoxic T-cells that recognize proteins expressed on stressed cells, and TNF. These additional mechanisms are discussed below.

The role of tumour necrosis factor in the immunopathogenesis of mycobacterial disease

As discussed above (see page 81), TNF-α makes a major contribution to protective immune responses in mycobacterial disease by inducing and sustaining granulomas. Paradoxically, though, it is also a key factor in tissue-destroying reactions leading to progression of disease. The reason for this is that macrophages and other cells infected with virulent strains of *M. tuberculosis* are rendered exquisitely sensitive to killing by TNF-α (Filley *et al.*,

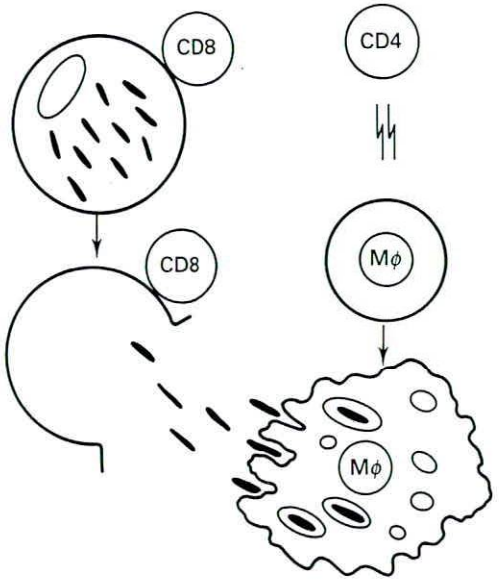

Fig. 5.5 The role of cytotoxic cells in protective immunity. The cytotoxic (usually CD8+) T-cell lyses a cell containing many mycobacteria in the cytoplasm, enabling them to be engulfed by an immunologically effective macrophage activated by CD4+ T-cells. Mϕ = macrophage

1992). This may well provide a means, in addition to cell-mediated cytotoxicity, by which tubercle bacilli are released from cells unable to control the intracellular infection.

In addition, infection by mycobacteria elicits a more generalized effect of TNF-α on tissues. This is analogous to the Shwartzman phenomenon, in which injection of a Gram-negative endotoxin into the skin primes the site for a necrotic reaction when the same endotoxin is given intravenously 24 hours later. The Koch phenomenon is somewhat similar because, as originally demonstrated by Koch (1891), systemic injection of tuberculin causes necrosis around tuberculous foci, with their elimination if they are superficial. Tumour necrosis factor-α is involved in the Koch phenomenon as the reaction is accompanied by a massive systemic release of this cytokine (Rook and Al Attiyah, 1991).

The Koch phenomenon elicited by mycobacteria differs in one important aspect from the Shwartzman phenomenon induced by Gram-negative endotoxin in that the former is T-cell dependent (Al Attiyah *et al.*, 1992). In addition, priming for necrosis requires cytokines from TH2, or a mixture of TH1 and TH2, T-cells. Cytokines from a pure TH1 T-cell population do not prime the tissues for necrosis (Fig. 5.6; Hernandez-Pando and Rook, 1994). This finding is of crucial importance as it explains how the T-cell maturation type, and the environmental and other factors determining the type, critically affect the nature of immune responsiveness to challenge by pathogenic mycobacteria and thereby the outcome of such a challenge. It also provided the

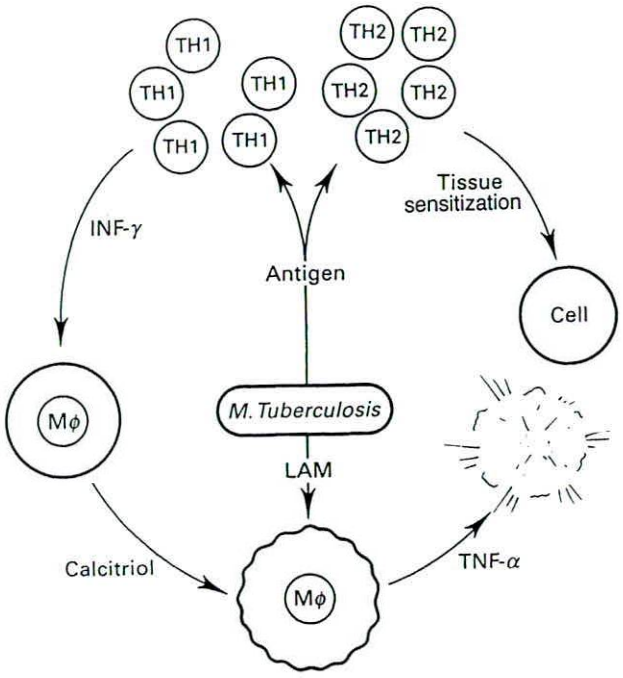

Fig. 5.6 Role of TH1 and TH2 cells in protection and immunopathology in tuberculosis. TH1 cells produce gamma interferon (IFN-γ) which initiates macrophage (Mϕ) activation (and granuloma formation). TH2 cells produce a substance which renders cells and tissues exquisitely sensitive to necrosis by tumour necrosis factor (TNF-α), the release of which from macrophages is triggered by mycobacterial lipoarabinomannan (LAM). Mϕ = macrophage

rationale for the development of successful immunotherapy for mycobacterial disease as described in Chapter 10.

Tuberculin and tuberculin reactivity

Koch's Old Tuberculin was a filtrate of a broth culture of *M. tuberculosis* concentrated by evaporation in a heated water bath. It contained various impurities derived from the medium and tended to induce non-specific inflammatory reactions. To overcome this problem Seibert (1934) attempted to harvest the tuberculoproteins by precipitation with acetone and ammonium sulphate. The resulting preparation was termed purified protein derivative of tuberculin (PPD) and has been used widely since. Despite the name, PPD is not pure protein.

Koch also produced New Tuberculins by grinding mycobacteria, thereby releasing their cytoplasmic antigens. This method of producing mycobacterial skin test reagents was reintroduced by Stanford and his colleagues. These reagents are now prepared by harvesting mycobacteria during the growing phase from non-antigenic media, washing them thoroughly, disrupting the cell

mass in an ultrasonicator, sterilizing the cytoplasm by repeated membrane filtration, and diluting it to a suitable protein concentration. The first of these reagents was Burulin, prepared for studies on Buruli ulcer in Africa (Stanford *et al.*, 1975), but subsequently they were prepared from many mycobacterial species (Editorial, 1984). New Tuberculins are relatively much richer in the species-specific antigens than PPD.

The two types of skin test reagents prepared from *M. leprae* (lepromins and leprosins) and the reactions elicited by them are described in Chapter 7 (see page 152).

A positive tuberculin reaction usually manifests as an area of induration which reaches a maximum after 48 or 72 hours. Erythema also occurs and may be much more extensive than the induration; but it is, by convention, ignored as it is difficult to see and measure in dark-skinned peoples. Some patients with active tuberculosis have visible reactions as early as six hours after testing (Kardjito and Grange, 1982). This early reaction is particularly prominent amongst healthy and radiologically clear persons who are occupationally exposed to patients with tuberculosis, suggesting that it is associated with protection (El Ansary and Grange, 1984; Grange *et al.*, 1986). Studies of punch biopsies of the early reaction show that it is a typical DTH reaction of early onset (Gibbs *et al.*, 1991).

The late component of the tuberculin reaction may also be caused by qualitatively different reactions. Stanford and Lema (1983) observed that some 48-hour tuberculin reactions in humans are purple coloured, indurated, well demarcated and tender, while others are pink, soft, ill-defined and much less tender. It has been postulated that these reactions correspond, respectively, to the necrotizing (Koch-type) and non-necrotizing reactions described in the mouse (see page 88). Blood flow studies based on the two types of reaction reveal considerable slowing of blood flow in the centre of the more indurated reactions – a phenomenon that could predispose to tissue necrosis (Potts *et al.*, 1992). It is possible that this central slowing of blood flow is due to an effect of TNF-α on the capillaries.

The histological appearance of a positive tuberculin test at 48 hours has been described in detail by Beck and his colleagues (1986, 1988) and is shown in Figs. 5.7 and 5.8. There is a dense infiltrate of mononuclear cells (lymphocytes and monocyte/macrophages) around the capillaries and skin appendages (sweat glands and hair follicles). In addition, some of these mononuclear cells migrate out of the perivascular and periappendicular foci and migrate towards the epidermis (Fig. 5.8). This migration, particularly that of the monocyte/macrophage cells, is greater in reactions to tuberculin than to leprosin in patients with tuberculosis, and *vice versa* in leprosy patients, suggesting that this component of the reaction is affected by species-specific antigens. The diameter of the reaction is not related to the intensity of the cellular infiltrate, measured as the area of the dermis occupied by the perivascular foci: some individuals with very large reactions have relatively few cells, while others who have no visible or palpable reaction have an intense cellular infiltrate. Thus the clinically evident features of the tuberculin reaction are almost certainly due to release of cytokines from the cells rather than to the cell mass itself.

The practical aspects of skin testing in epidemiological studies and for the diagnosis of leprosy and tuberculosis are discussed in Chapters 6, 7 and 8, respectively.

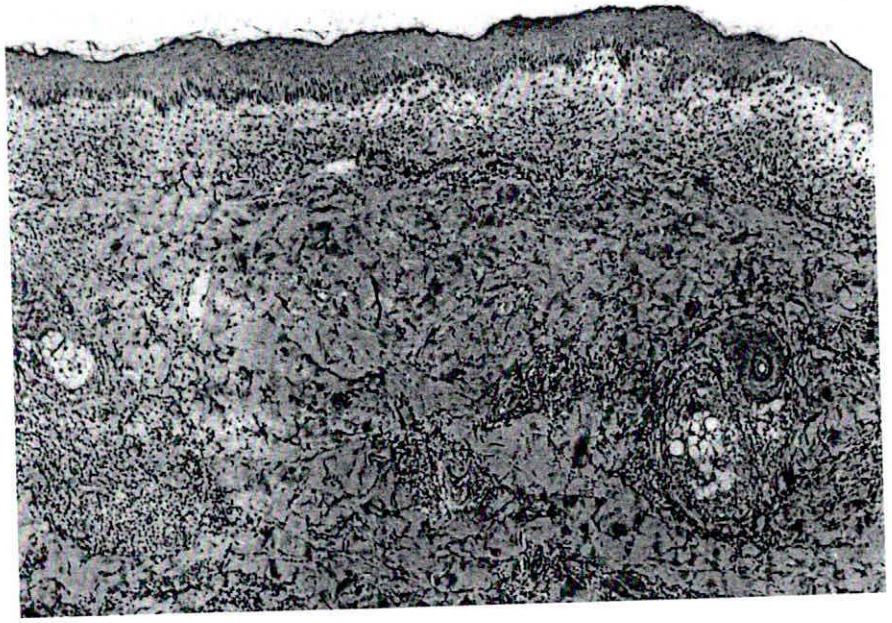

Fig. 5.7 Thin section of a biopsy of a tuberculin reaction at 48 hours, showing perivascular and periappendicular foci of inflammation. Courtesy of Prof. J. Swanson Beck

Categories of tuberculin reactors

Multiple skin testing with a range of reagents (New Tuberculins, see above) prepared from different species has shown that individuals sensitized by contact with mycobacteria may be placed in three distinct categories according to their patterns of reactivity (Stanford *et al.*, 1981a). Category 1 individuals react to any New Tuberculin, indicating that they respond to the shared (Group i) mycobacterial antigens. Category 2 individuals react to none of the reagents and this may be a genetic feature associated with HLA-D (Class II) histocompatibility antigens (see page 107). There is no evidence that such non-responders are unable to develop protective immunity; indeed, as mentioned above, an intense cellular infiltrate may occur in the absence of clinically evident reactivity. Category 3 individuals react to some but not all reagents, indicating that they are responding to the species-specific (Group iv) mycobacterial antigens.

Three important points arise from this categorization. First, an awareness of the category 2 non-responders is relevant to the use of BCG. Some unfortunate non-responders have been repeatedly and unnecessarily skin tested and revaccinated in attempts to make them convert. The presence of a BCG scar is sufficient evidence that the vaccination has 'taken'. Second, surveys of the

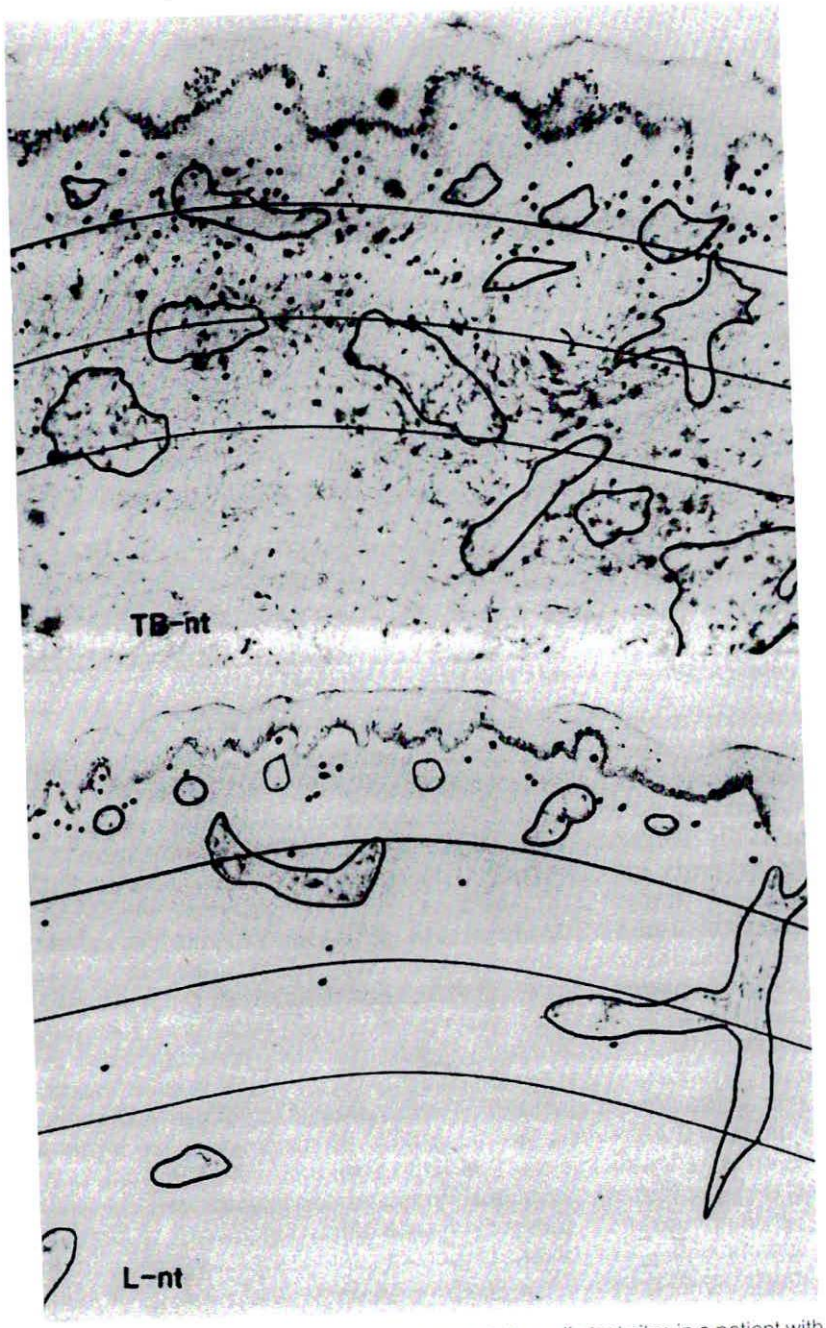

Fig. 5.8 Thin sections of biopsies of the 48-hour tuberculin test sites in a patient with tuberculosis tested with tuberculin (TB-nt) and leprosin (L-nt) showing the difference in intensity of the cellular reaction in the homologous and heterologous reactions. The perivascular infiltrates and mononuclear cells in the dermis, particularly in the subepidermal zones, are marked. Courtesy of Prof. J. Swanson Beck

reactivity of the category 3 responders is a useful way of determining which mycobacteria are present in an environment without resorting to the time-consuming procedure of isolating and identifying strains from inanimate sources. Third, there is an interesting relation between categorization and disease. Studies on leprosy patients in Nepal (Stanford *et al.*, 1981a) and on tuberculosis patients in Indonesia (Kardjito *et al.*, 1986) both showed that most healthy reactive persons are category 1 reactors while the majority of patients with either disease are category 3 reactors (Fig. 5.9).

In order to understand the relevance of this reduced responsiveness to common mycobacterial antigens to the development and pathogenesis of mycobacterial disease, it is necessary to consider the nature of the protective epitopes of mycobacteria.

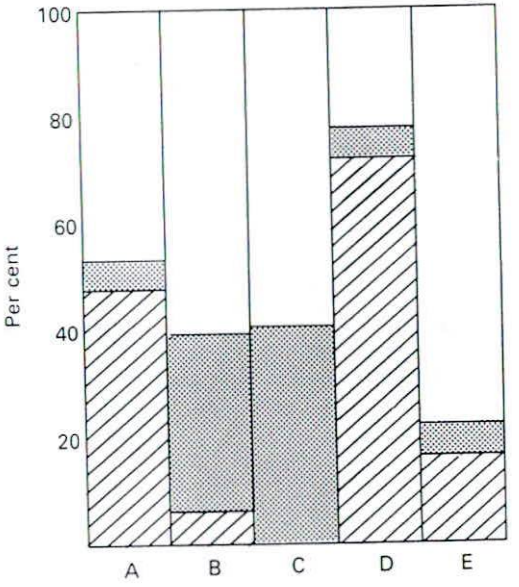

Fig. 5.9 Distribution of the three categories of tuberculin reactors amongst (A) healthy Nepalese individuals, (B) Nepalese patients with tuberculoid leprosy, (C) Nepalese patients with lepromatous leprosy, (D) healthy Indonesian individuals, and (E) Indonesian patients with pulmonary tuberculosis. Hatched: category 1; grey: category 2; white: category 3 reactors. Data from Stanford *et al.* (1981a) and Kardjito *et al.* (1986)

Protective and cross-protective antigens

It is a widespread belief that 'protective antigens', i.e. those that elicit an effective immune response, are always species- or strain-specific and that vaccines are ineffective unless they contain such antigens. This is certainly true when the determinant of virulence is a strain-specific toxin or a viral receptor site. On the other hand, there is no reason to assume that it is necessarily the case with intracellular pathogens such as the mycobacteria. Indeed, the finding that BCG is as protective against leprosy as it is against tuberculosis

(Brown *et al.*, 1966), and the evidence that it protects children against cervical lymphadenitis due to *M. avium* (Trnka *et al.*, 1994), strongly indicate that the protective epitopes are to be found among those common to all mycobacteria.

As outlined in Chapter 2, some soluble mycobacterial antigens are actively secreted by viable bacterial cells. These are probably the first to be presented to CD4+ T-cells by Class II MHC molecules and could therefore be of particular relevance to protection involving macrophage activation and granuloma formation. On the other hand, cytolytic activity is crucial to protection and may be induced by a different type of antigen. In this context, a class of proteins known as the heat-shock proteins (HSPs) are of particular relevance. Heat-shock proteins are present in all living cells and are structurally highly conserved. Thus there are many epitopes common to HSPs from mycobacteria and mammals. Heat-shock proteins are normally present in small amounts in the cytoplasm and are involved in the assembly and shaping of newly synthesized proteins, hence their alternative name *chaperonins* or nurse-maid proteins. Under conditions of stress, such as heat shock, they are over-produced and expressed on the surface of the cell (Born *et al.*, 1990).

Mycobacterial HSPs may thus be presented on the surfaces of infected cells but, in addition, intracellular infection, particularly uncontrolled infection, may stress the cell leading to over-production and expression of 'self' HSPs. Whatever the origin, HSPs on the cell surface may act as targets for cytolytic cells. Indeed a population of CD4+ cytolytic cells recognizing the mycobacterial 65 kDa HSP have been described (Ottenhoff *et al.*, 1988). In addition, a major subpopulation of γ-δ cells recognize HSPs on cell surfaces and may therefore destroy stressed, infected macrophages and other cells expressing HSPs on their surfaces (Orme *et al.*, 1993). As mentioned above (see page 85), these cells may also be required for activation of CD4+ T-cells. It is therefore likely that mycobacterial HSPs play a central role in protective immunity. It is in this context that the reduced recognition of common mycobacterial antigens in patients with mycobacterial disease could be relevant as HSPs are major components of this group of shared antigens.

There is evidence that diminished dermal reactivity to common mycobacterial antigens is not just a feature of mycobacterial disease but occurs in other conditions characterized by intracellular parasitism, such as human immunodeficiency virus (HIV) infection (Khoo *et al.*, 1993) and South American trypanosomiasis (Chagas' disease; Bottasso *et al.*, 1994). Thus immunotherapy designed to induce or restore immune recognition of widely distributed antigens, which probably are, or include, HSPs might be effective in a range of unrelated conditions, both infectious and neoplastic, in which recognition and lysis of abnormal, stressed cells is central to protection (Grange *et al.*, 1995).

Vaccination strategies

Many attempts were made to prepare a vaccine against tuberculosis in the years immediately following Koch's discovery of the tubercle bacillus. As a result of work by Koch and Trudeau it became a generally accepted dogma that an effective vaccine would be a live attenuated one rather than a killed

one. Calmette and Guérin eventually produced such a vaccine from a strain isolated from a case of bovine mastitis, and therefore presumed to be *M. bovis*, by passaging it 230 times over a period of 11 years, by which time extensive animal studies showed it to be stably attenuated. These workers prepared their vaccine from a bovine rather than a human isolate on account of a belief that self-limiting tuberculous lesions due to *M. bovis* in childhood afforded protection against pulmonary tuberculosis later in life (Marfan's Law). This vaccine, Bacille Calmette–Guérin (BCG), was originally given orally to neonates to mimic infection by milk containing *M. bovis* but problems of safety and standardization later led to to the introduction of the present freeze-dried form given by intracutaneous injection or multipoint inoculation.

The protective efficacy of BCG vaccine has been the subject of considerable controversy. The results of a number of major vaccine trials (Table 5.2) show that protection varies from 80 per cent to none at all. The probable reason for this variation is discussed above (see page 88). When given to an uninfected (tuberculin negative) child, BCG confers protection against the serious consequences of subsequent primary infection, such as tuberculous meningitis. On the other hand, it is much less effective in preventing tubercle bacilli from persisting in the tissues and causing post-primary tuberculosis later in life. Thus the vaccine should not be given to infected persons, indeed it is liable to cause severe reactions if given to tuberculin reactors.

In view of the inadequacies of BCG vaccine, there is much interest in using DNA recombinant technology to develop new vaccines. One suggested approach is to identify the determinants of virulence in *M. tuberculosis* and to delete them by, for example, transposon mutagenesis, but this approach would probably achieve no more than reinventing BCG. Another approach is to identify protective antigens and to introduce multiple copies of these into BCG or an alternative vector such as vaccinia.

A quite different approach to vaccination is to use an environmental mycobacterium that never causes disease, presumably because it is only capable of eliciting protective responses. In this context, BCG, although attenuated, is still capable of causing severe local reactions if given to tuberculin-

Table 5.2 Results of nine major BCG vaccine trials

Region	*Year of commencement*	*Age range*	*Protection afforded* (%)
North America*	1935	0–20 years	80
Chicago, USA	1937	3 months	75
Georgia, USA	1947	6–17 years	0
Illinois, USA	1948	Young adults	0
Puerto Rico	1949	1–18 years	31
Georgia, USA	1950	5 years	14
Great Britain	1950	14–15 years	78
South India	1950	All ages	31
South India	1968	All ages	0**

* Amerindian population
** A later follow-up revealed some protection in those vaccinated in infancy

positive persons. *Mycobacterium vaccae*, a rapidly growing non-pathogen, can safely be given to such persons and there is accumulating evidence that it is an effective immunotherapeutic agent for active tuberculosis (see Chapter 10). If able to induce the immune-mediated destruction of persisting mycobacteria in overt disease it should, by analogy, do so before reactivation occurs and it is thus a candidate vaccine (Stanford and Grange, 1993).

Attempts have also been made to prepare vaccines against leprosy but, as the causative organism cannot be cultivated *in vitro*, it is not possible to prepare a living attenuated vaccine from this species. Alternative approaches include adding killed armadillo-derived *M. leprae* to BCG and using environmental mycobacteria as vaccines. At present, BCG itself is used as a vaccine against leprosy and high levels of protection have been observed in regions where it also protects against tuberculosis.

Immunological spectra in mycobacterial disease

The clinical features and course of a mycobacterial disease are, in a very large measure, dependent upon the immunological reactivity of the patient. Consequently, the concept of a 'spectrum' of such reactivity, from highly active at one pole to absent at the other, has been developed. At first view this appears a reasonable and attractive idea but, as in most areas of mycobacterial immunology, the matter is not as straightforward as it first seems to be.

The immune spectrum in leprosy

The great variation in the appearance and behaviour of the determinate forms of leprosy results from their position on an immunopathological spectrum described in detail by Ridley and Jopling (1966). For convenience, five points on the spectrum are recognized: the two polar forms, tuberculoid (TT) and lepromatous (LL), and three intermediate points, borderline tuberculoid (BT), mid-borderline (BB) and borderline lepromatous (BL). The clinical, immunological and histological features of these forms are shown in Table 5.3 and are described in more detail in Chapter 7.

Table 5.3 Characteristics of the five points in the immunological spectrum of leprosy

Characteristic	*Point on the spectrum*				
	TT	BT	BB	BL	LL
Bacilli in lesions	–	±	+	++	+++
Bacilli in nasal discharge	–	–	–	+	+++
Granuloma formation	+++	++	+	–	–
In vitro correlates of CMI	+++	++	+	±	–
Reaction to lepromin	++	++	–	–	–
Anti *M. leprae* antibodies	±	±	+	++	+++
Macrophage maturity	mature ←				→ immature
Response to therapy	good ←				→ poor

At first view, the tuberculoid pole appears to be characterized by effective immunity which then decreases across the spectrum to the lepromatous pole where there is no apparent protective immune reactivity. Thus the lesions of tuberculoid leprosy are characterized by very few bacilli, many lymphocytes and granulomas containing mature epithelioid cells. In contrast, lesions of lepromatous leprosy contain few lymphocytes but numerous bacilli within immature macrophages. Thus, patients with tuberculoid leprosy are sometimes regarded as being near normal and those with lepromatous disease as being the most abnormal. For this reason, shifts in the position of the disease towards the tuberculoid and lepromatous poles of the spectrum are, respectively, termed 'upgrading' and 'downgrading'. An alternative view is that all forms of determinate leprosy are equally abnormal, but that the nature of the abnormality differs. Although tuberculoid leprosy is often self-limiting, the granuloma-forming immune response appears to be greatly out of proportion to the amount of antigen present. It may, in a large part, be a hyper-reactive response to bacterial debris that is not readily cleared from the lesion. The mechanism of effective immunity to leprosy must be sought in those contacts who either never develop the disease or display self-limiting indeterminate lesions.

The factors that determine whether an infected person will develop leprosy and, if so, in what form are poorly understood. It was originally considered that the outcome of infection is related to the time taken for CMI to develop (Godal *et al.*, 1974). Thus, in healthy contacts, a rapid onset of CMI would eliminate the bacilli before lesions had a chance to develop. In tuberculoid leprosy a slight delay would permit enough multiplication of the bacilli to render their removal more difficult, while an indefinite delay in the onset of CMI would result in lepromatous leprosy. The alternative, and more widely accepted, view is that the type of disease is 'predestined' by various factors, including genotypes and prior exposure to environmental mycobacteria (see page 88), and is the result of balances between the various T-cell subsets that modulate and control immune reactivity.

One of the most extraordinary features of leprosy is the total lack of immune responsiveness to *M. leprae* in the lepromatous form of the disease. This lack of responsiveness is highly specific as patients can respond, sometimes very strongly, in skin tests to tuberculin and other mycobacterial reagents (Stanford, 1994). The defect appears to be due to a suppression of the activity of *M. leprae*-specific CD4+ T-cells rather than to an absence of these cells (Bloom *et al.*, 1992). *In vitro* lymphocyte transformation tests show that T-helper cells from LL patients respond well to purified antigens of *M. leprae* but not to whole bacilli, suggesting that the latter are able, directly or indirectly, to suppress T-helper cell activation.

The immunological spectrum in leprosy is related to the Type 1 and Type 2 maturation pathways of T-cells. Thus in TT leprosy the *M. leprae*-specific T-cells in the blood are mostly of the TH1 type and TH1-associated cytokines are detectable in the lesions. In LL leprosy the cytokine response shows a mixed Type 1 and Type 2 pattern (Yamamura *et al.*, 1991). Thus, in both TT leprosy and tuberculosis, a TH1-mediated response is associated with protective immunity but the effects of the mixed TH1/TH2 responses differ in the two diseases. In tuberculosis, this mixed response leads to tissue-necrotizing

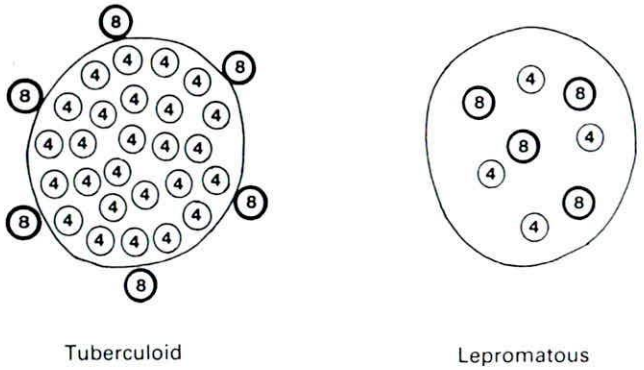

Fig. 5.10 The distribution of CD4+ (putative helper phenotype) and CD8+ (putative suppressor phenotype) T-cells in lesions of tuberculoid and lepromatous leprosy. Data from Modlin *et al.* (1983)

hypersensitivity but this does not occur regularly in leprosy. This appears to be the result of a strong and specific suppression of immune reactivity: clones of CD8+ T-cells from lepromatous lesions are able to suppress proliferation of CD4+ T-cells (putative helper phenotype) specific for *M. leprae* (Bloom, 1986). Immunocytological studies on the lesions of tuberculoid and lepromatous leprosy (Fig. 5.10) have shown that there are many T-cells in tuberculoid lesions but that CD8+ T-cells (putative suppressor phenotype) are in a minority and occur on the outside of the granuloma. In lepromatous lesions there are relatively few T-cells, but a much higher proportion of these are of the CD8+ type and are found within rather than around the lesion (Modlin *et al.*, 1983).

The ability of LL patients to react strongly to tuberculin while not responding to *M. leprae* which shares many epitopes with *M. tuberculosis* suggests that there are two types of immune defect in these patients. First, there is a failure to respond to common (Group i) mycobacterial antigens, as is seen in tuberculosis and some other diseases characterized by intracellular parasitism (see page 95). Secondly, there is a defect in response to the species-specific (Group IV) epitopes of *M. leprae* but not to such epitopes in other mycobacteria which may thus elicit responses in skin testing and other tests for cell-mediated immune reactions. (As noted in Chapter 2, *M. leprae* is one of a small group of mycobacteria that possess only Group i and iv antigens: they lack the Group ii and iii antigens that characterize the slow and rapid growers respectively.) The skin-test reactivity in patients with tuberculosis and with tuberculoid and lepromatous leprosy is summarized in Table 5.4.

Further light on the immune defect in leprosy is shed by studies on the so-called leprosy reactions, of which there are two types described in detail in Chapter 7. Type 1 reactions occur in patients with borderline forms of leprosy (BT, BB, BL) and are often associated with a shift in immunity towards the TT pole. The cytokines detected in Type 1 reactions are predominantly TH1-induced, suggesting that the reactions are associated with a TH2 to TH1 shift (Stanford, 1994). Type 2 reactions, erythema nodosum leprosum (ENL), occur in patients at or near the LL pole. The cytokines in the lesions are of a

Table 5.4 Skin test reactivity to leprosin and tuberculin in healthy individuals and those with tuberculoid (TT) and lepromatous (LL) leprosy and with tuberculosis all of whom have been sensitized to both *M. leprae* and *M. tuberculosis*

Group	*Reagent*	*Antigen groups in reagent**	*Antigen groups to which reactivity is suppressed*	*Reaction*
Health	Leprosin	i,iv(L)	None	Positive
	Tuberculin	i,ii,iv(T)	None	Positive
TT leprosy	Leprosin	i,iv(L)	i	Positive
	Tuberculin	i,ii,iv(T)	i	Positive
LL leprosy	Leprosin	i,iv(L)	i,iv(L)	Negative
	Tuberculin	i,ii,iv(T)	i	Positive
Tuberculosis	Leprosin	i,iv(L)	i	Positive
	Tuberculin	i,ii,iv(T)	i	Positive

* i = common to all mycobacteria; ii = slow grower specific (absent from *M. leprae*); iv(L) antigens specific to *M. leprae*; iv(T) antigens specific to *M. tuberculosis* (see Fig. 2.1, page 13)

mixed Type 1 and Type 2 pattern, reflecting the underlying leprosy-specific T-cell maturation pattern at this end of the spectrum. Though ENL is usually said to be an example of an antigen–antibody complex reaction, TNF-mediated tissue necrosis is involved and is probably the initiating event. This appears to be due to a relaxation of suppression by CD8+ cells (Filley *et al.*, 1989).

Although details are far from clear, it appears that LL leprosy patients are protected against tissue-necrotizing hypersensitivity of the Koch type by the specific CD8+ T-cell-mediated suppression described above. On the other hand, they lack the pure TH1 cytokine response that would lead to granuloma formation, bacillary destruction and ultimate resolution of the disease. In this respect, injection of microgram amounts of gamma interferon (a TH1 cytokine) into the skin of LL patients leads to an increase in the number of CD4+ cells relative to CD8+ cells, granuloma formation and destruction of leprosy bacilli, indicating a shift towards the TT pole of the spectrum (Samuel *et al.*, 1987).

For more details on the spectrum and immunopathology of leprosy see Ridley (1988).

The immune spectrum in tuberculosis

In view of the description of the immune spectrum in leprosy, the existence of a similar spectrum in tuberculosis has been postulated by several workers. Ridley and Ridley, for example, divided 54 patients with tuberculosis into three histological groups, each with two subgroups (Table 5.5; Ridley and Ridley, 1987). In common with leprosy, immune reactivity, including the maturity of the macrophages in the lesions, and the bacillary load are inversely related

Table 5.5 The spectrum of tuberculosis. Data from Ridley and Ridley (1987)

Group	Principal cell type	Necrosis	Giant cells	Bacilli
1a	Organized mature epithelioid cells	None	+	None
1b	Unorganized mature and immature epithelioid cells	Patchy fibrinoid	+	Rare
2a	Immature epithelioid cells	Caseation, no nuclear debris	+	Scanty
2b	Immature epithelioid cells and/or undifferentiated histiocytes	Necrosis with nuclear debris and polymorphs	+	+
3a	Scanty macrophages	Extensive, basophilic; coarse nuclear debris	–	++
3b	Very few macrophages	Extensive, eosinophilic; scanty nuclear debris	–	+++

Despite these similarities, there are considerable differences between the spectra of tuberculosis and leprosy (Skinsness, 1968). Tuberculosis may be associated with immunological anergy but the specific defect seen in lepromatous leprosy does not occur. Instead, anergy in tuberculosis is usually more generalized and due to some unrelated cause such as HIV infection (see below). Although CD8+ cells are found in lesions of severe, progressive tuberculosis (Ainslie *et al.*, 1992), it is uncertain whether they are a cause or a result of diminished immune responsiveness.

Two classes of skin reactions occur in tuberculosis, tuberculides and erythema nodosum, and they bear a superficial resemblance to the Type 1 and Type 2 leprosy reactions respectively. The term tuberculide covers a number of skin lesions which are divisible into two main types: lichen scrofulosorum and papulonecrotic tuberculide. The former reactions commence as perivascular and periappendicular infiltrations of white cells in the dermis and develop into non-necrotic granulomas with epithelioid and giant cells, somewhat resembling the lesions of tuberculoid leprosy. In the latter there is necrosis due to an obliterative vasculitis which, on occasions, is very extensive. The pathogenesis of both types is unknown but they may be due to exaggerated non-necrotizing and necrotizing hypersensitivity reactions to blood–borne tubercle bacilli, either whole or fragmented.

Erythema nodosum is a nodular vasculitis affecting the subdermal connective tissue and may occur in several infectious diseases, especially those caused by streptococci. As a manifestation of tuberculosis, it usually occurs in children with primary infection at the time of tuberculin conversion and it may be associated with phlyctenular conjunctivitis. In a rare chronic form, erythema induratum or Bazin's disease, the lesions may ulcerate. The pathogenesis of this reaction, and its relation to erythema nodosum leprosum, is not

The immune spectrum in *Mycobacterium ulcerans* infection

Immunological reactivity in this relatively uncommon disease is bizarre and fascinating. The course of the disease is shown in Fig. 5.11. The lesion, more fully described in Chapter 9, commences as a skin nodule that may either resolve or progress to overt ulceration. Unlike other pathogenic mycobacteria, a major determinant of the virulence of *M. ulcerans* is a toxin which, in the progressive cases, causes widespread necrosis and liquefaction of the subcutaneous fat (Hockmeyer *et al.*, 1978). Secondary necrosis of the overlying skin results in deeply undermined ulcers, often reaching enormous sizes. During the progressive ulcerative stage there is evidence of immunological anergy as the lesions contain many bacilli but there is little or no cellular response and the patient fails to react to Burulin, a skin test reagent prepared from the causative organism (Stanford *et al.*, 1975). The anergy is less specific than in lepromatous leprosy as patients fail to react to tuberculin as well as to Burulin. There is some evidence that this anergy is due to trapping of T-cells responding to mycobacterial antigen in the lymph nodes.

Unless the lesion is excised, a stage is reached when an effective immune response ensues, lymphocytic infiltrates and granuloma formation are seen, the bacilli decline in number and then disappear, the patient reacts to Burulin and tuberculin and the lesion eventually heals by fibrosis. The cause of the characteristic shift from anergy to immune reactivity in this disease is not understood. It appears to be a local phenomenon as a lesion may show anergy in one part and healing in another. It would be of interest to determine whether this shift is due to changes in local cytokine patterns related to TH2 and TH1 T-cells.

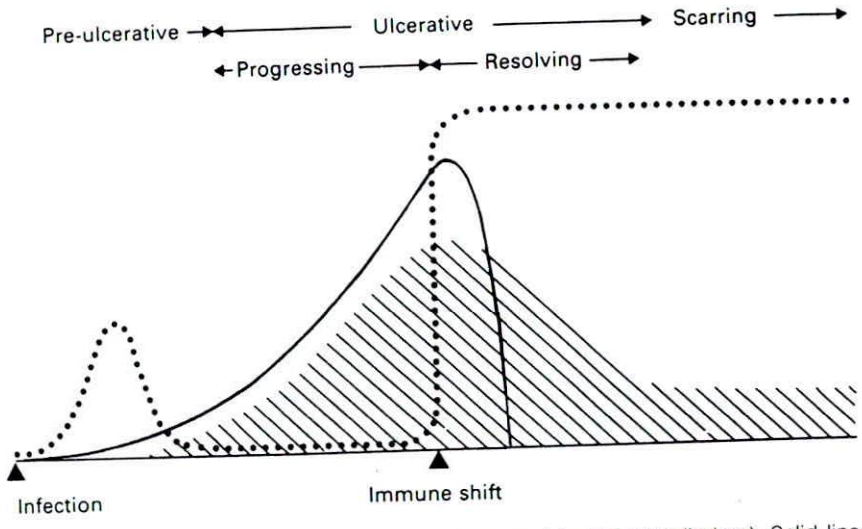

Fig. 5.11 The time course of *Mycobacterium ulcerans* infection (Buruli ulcer). Solid line: bacillary load; dotted line: reactivity to Burulin and correlates of cell-mediated immunity. The shaded area indicates the extent of clinically evident lesion

Immunotherapy

Many attempts have been made to treat tuberculosis and leprosy by stimulating the patient's immune reactivity. Koch's use of Old Tuberculin was the first such attempt and it appeared to be of value in the case of skin tuberculosis. Many other attempts followed, with some claims of success.

Until recently, attempts at immunotherapy for mycobacterial disease were mostly based on non-specific stimulation of immune responsiveness by means of mycobacterial antigens or immunostimulating drugs such as levamisole. As it is now clear that there are fundamental differences between protective and tissue-necrotizing immune responses, it is important that immunotherapy should enhance the former and, hopefully, suppress the latter. Following observations that BCG is very effective as a vaccine against leprosy in parts of Uganda and that the efficacy of this vaccine is affected by prior sensitization of the population by environmental mycobacteria, a number of these species were isolated from Ugandan mud and extensively studied. One species, *M. vaccae*, was found to down-regulate the Koch phenomenon and to restore skin test reactivity to the common mycobacterial antigens (Stanford *et al.*, 1994). Although originally intended as a leprosy vaccine, it was found to be a useful agent for the immunotherapy of tuberculosis as described in Chapter 10. This immunotherapeutic agent is a TH1 adjuvant and thus induces protective cell-mediated immune reactions and, by restoring recognition of common antigens including heat-shock proteins, it probably also facilitates recognition and killing of stressed, mycobacteria-laden cells. It is likely that all these mechanisms are secondary to shifts in the steroid hormone balance in the lymphoid microenvironment (Rook *et al.*, 1994; see page 89).

Humoral factors and serodiagnosis

It is unlikely that antibody plays a significant part in the immune response to mycobacteria. Children with disseminated tuberculosis have lower levels of antibody to mycobacterial lipoarabinomannan (LAM) than those with localized disease but the relation between this antibody response and pathogenesis is not clear (Costello *et al.*, 1992). Other correlations between antibody levels and disease status may merely reflect the antigen load or the maturation pathway of the mycobacteria-specific T-cells as TH2 cells facilitate antibody production but TH1 cells do not.

A serological characteristic of active tuberculosis and some autoimmune diseases characterized by tissue necrosis is an elevated level of agalactosyl immunoglobulin G (Gal(0); Rook, 1988). This is immunoglobulin G (IgG) which lacks the terminal galactose on the sugar chain in the CH_2 domain of the heavy chain. It accounts for 20–25 per cent of the total circulating IgG, rising to 40 per cent or more in the elderly. Its function is unknown but it appears to be an indicator of Koch-type tissue necrotizing reactions and its decline is a useful marker of the efficacy of immunotherapy for tuberculosis (Rook *et al.*, 1994).

Though of limited relevance to pathogenesis, there have been numerous [illegible] for diagnostic purposes

(Grange, 1984; Wilkins, 1994). Despite a huge amount of effort, no serodiagnostic test is in widespread clinical use. The reason for this is that, although antibodies are undoubtedly produced in mycobacterial disease, the overlap between levels in patients and either healthy individuals or those with other diseases is unacceptably large. 'Natural' antibodies are probably due to contact with mycobacteria and related genera in the environment, but the use of purified specific antigens in diagnostic tests has proved disappointing: most of the humoral immune response is directed towards shared antigens. The introduction of monoclonal antibodies made it possible to develop competition assays for antibody to specific epitopes. Although this approach led to high specificity (i.e. very few 'false positives'), the sensitivity (the ability to diagnose disease when present) was rather low (Wilkins, 1994), although the use of a set of several monoclonal antibodies, while making the test rather complex, improved the sensitivity (Hoeppner *et al.*, 1987).

Serodiagnosis of leprosy has been attempted by using the PGL-1 antigen but, as is explained in Chapter 7, only patients with multibacillary disease have antibody levels significantly higher than those of healthy contacts.

The use of antibody to detect mycobacterial antigen, and antigen–antibody complexes, in clinical specimens has not received as much attention as serodiagnosis although the results of a limited number of studies are encouraging, with high sensitivity and specificity. In general, the best results have been obtained with 'clean' specimens such as cerebrospinal, pleural and peritoneal fluids (Wadee *et al.*, 1990).

Mycobacterial adjuvants

An adjuvant is a substance that enhances the antibody response to unrelated antigens administered with it. One of the best known is Freund's complete adjuvant which consists of a water-in-oil emulsion containing killed tubercle bacilli. The adjuvant activity of mycobacteria resides principally in the peptidoglycan (murein) of the cell wall, although trehalose dimycolate (cord factor), RNA and certain peptidoglycolipids also appear to possess this activity. In the case of peptidoglycan, the minimum structure required for adjuvant activity is muramyl dipeptide (Chapter 2, page 17), a water soluble molecule that has been synthesized.

Despite many studies, the mode of action and natural function of adjuvants remain shrouded in mystery. Reactions to them may be associated with a primitive immune recognition system which responds to certain common microbial components. In addition to enhancing antibody production, adjuvants affect T-cells and can thereby cause some degree of macrophage activation. Indeed this property might be of relevance to the containment of mycobacterial infections before the onset of specific cell-mediated immunity as muramyl dipeptide induces granulomas very similar in appearance to those of immune origin in tuberculosis. Theories as to the mode of action of adjuvants include assistance in antigen presentation to T-cells, effects on lymphocyte traffic and non-specific mitogenic effects on B-cells. They may also act by modulating the steroid hormone balance in the lymphoid microenvironment (see page 89).

Mycobacterial disease and autoimmunity

There has been considerable interest in a possible relationship between infection by mycobacteria and autoimmune disease. Freund's complete adjuvant (a suspension of killed mycobacteria in oil: see above) induces autoimmune arthritis in rats. Some patients with tuberculosis develop a sterile arthritis (Poncet's disease; see page 174), although this is uncommon.

There are some other fascinating links between mycobacterial and autoimmune diseases (Rook and Stanford, 1992). Thus, in common with tuberculosis, patients with rheumatoid arthritis (RA) have elevated levels of agalactosyl IgG (Rook, 1988). Also, patients with RA are often of the HLA-DR4 phenotype which is associated with strong dermal reactivity to species-specific antigens of *M. tuberculosis* (see below; page 107).

As mycobacterial heat-shock proteins (HSPs) share many epitopes with their human analogues they could, in theory, induce autoimmune phenomena (Das and Grange, 1993). Indeed the arthritis inducible in rats by Freund's complete adjuvant is adoptively transferable by T-cells reacting to the mycobacterial 65 kDa HSP (van Eden *et al.*, 1988). In practice, there is no firm evidence that HSPs or other mycobacterial components induce autoimmune phenomena that contribute significantly to immunopathology and it has been postulated that they are usually prevented from doing so by an elaborate regulatory system (Cohen and Young, 1991).

Genetic factors in mycobacterial immunity

As only a minority of those infected with mycobacteria develop overt disease, many attempts have been made to establish some genetic marker of susceptibility or resistance. Studies on identical and non-identical twins strongly suggest an inherited predisposition or resistance to tuberculosis and racial variations have been suggested. Extensive studies in the mouse have revealed that an allele, designated *Bcg*, confers natural macrophage-mediated resistance to a range of intracellular pathogens including BCG, salmonellae and *Leishmania donovani* (Skamene *et al.*, 1982). This allele codes for a protein termed natural-resistance-associated macrophage protein (*Nramp*) which is involved in the generation of reactive nitrogen intermediates (Vidal *et al.*, 1993). A human homologue of this protein has been found but its significance to protection is unknown. As doubt has been shed on the ability of human macrophages to generate sufficient levels of reactive nitrogen intermediates to kill mycobacteria (see page 82), this gene may not play such a key role in resistance to disease in man as it does in mice.

Many attempts have been made to link susceptibility of tuberculosis and leprosy to the Class I antigens (HLA-A and HLA-B) of the major histocompatibility complex. Although some studies show a low but significant association of a particular HLA type to overt disease, the results vary from region to region and no definite pattern has emerged. In the case of Class II (HLA-D) antigens, it has been shown that HLA-DR2, particularly the DR15 subtype, predisposes to the development of tuberculosis, particularly radiologically-

et al., 1991; Khomenko *et al.*, 1990). The HLA-DR2 specificity may affect antigen recognition as individuals of this genotype have higher levels of antibody to epitopes on a 38 kDa protein unique to *M. tuberculosis* than those lacking this genotype (Bothamley *et al.*, 1989).

In leprosy, HLA-DR3 predisposes to tuberculoid leprosy and protects against lepromatous leprosy while HLA-DQw1 predisposes to the development of lepromatous leprosy (Ottenhoff and de Vries, 1987).

Class II types also affect skin test responses to mycobacterial antigens. Sensitized individuals who, nevertheless, do not respond to testing with such antigens (category 2 non-responders; see page 93) do not express the HLA-DR3 specificity (van Eden *et al.*, 1983) while those of HLA-DR4 phenotype respond relatively strongly to species-specific antigens of *M. tuberculosis* but not to such antigens of other mycobacterial species (Ottenhoff *et al.*, 1986). Larger tuberculin reactions were also found in HLA-DR15-positive than in DR15-negative tuberculosis patients (Bothamley *et al.*, 1995).

Mycobacterial disease and immunosuppression

Immunosuppression of whatever cause predisposes to the development of tuberculosis, whether due to primary infection or to endogenous reactivation or exogenous reinfection. Since the early 1980s, HIV has become an increasingly common predisposing factor in tuberculosis as described in detail in Chapter 8. In addition, HIV infection predisposes to disease due to environmental mycobacteria, notably the *M. avium* complex (see below and Chapter 9).

There is no doubt that HIV infection has a profound effect on tuberculosis and there is evidence that the latter disease adversely affects the course of the former. It has been observed that, even if successfully treated, tuberculosis in an HIV-positive person has a very deleterious effect on future health (see Chapter 6). Indeed, tuberculosis appears to drive the patient into the full picture of AIDS with considerable shortening of life. While the details are not clear, there is evidence that TNF-α and other immunological mediators released in tuberculosis lead to transactivation of the HIV provirus and its subsequent replication (Osborn *et al.*, 1989). In addition, tuberculosis causes a CD4+ T-cell lymphopenia which may add to that induced by the HIV (Beck *et al.*, 1985). Whatever the cause, the occurrence of active tuberculosis in the HIV-positive patient has very serious consequences, demanding strenuous efforts to prevent such disease by programmes of chemoprophylaxis or, preferably, immunoprophylaxis.

Not only is there a reduced ability to develop the characteristic high turnover granuloma of immunogenic origin in immunosuppressed tuberculosis patients, there is also a suppression of tissue-necrotizing reactions and scar formation that would otherwise limit the spread of infection. Thus, discrete pulmonary lesions and cavity formation are both less common in such patients. Instead, there may be radiologically rather non-specific spreading pulmonary lesions (Fig. 5.12). Non-pulmonary lesions due to unrestricted bacillary dissemination are frequent in such patients, particularly in the more profoundly immunosuppressed, and may present as one or more solitary

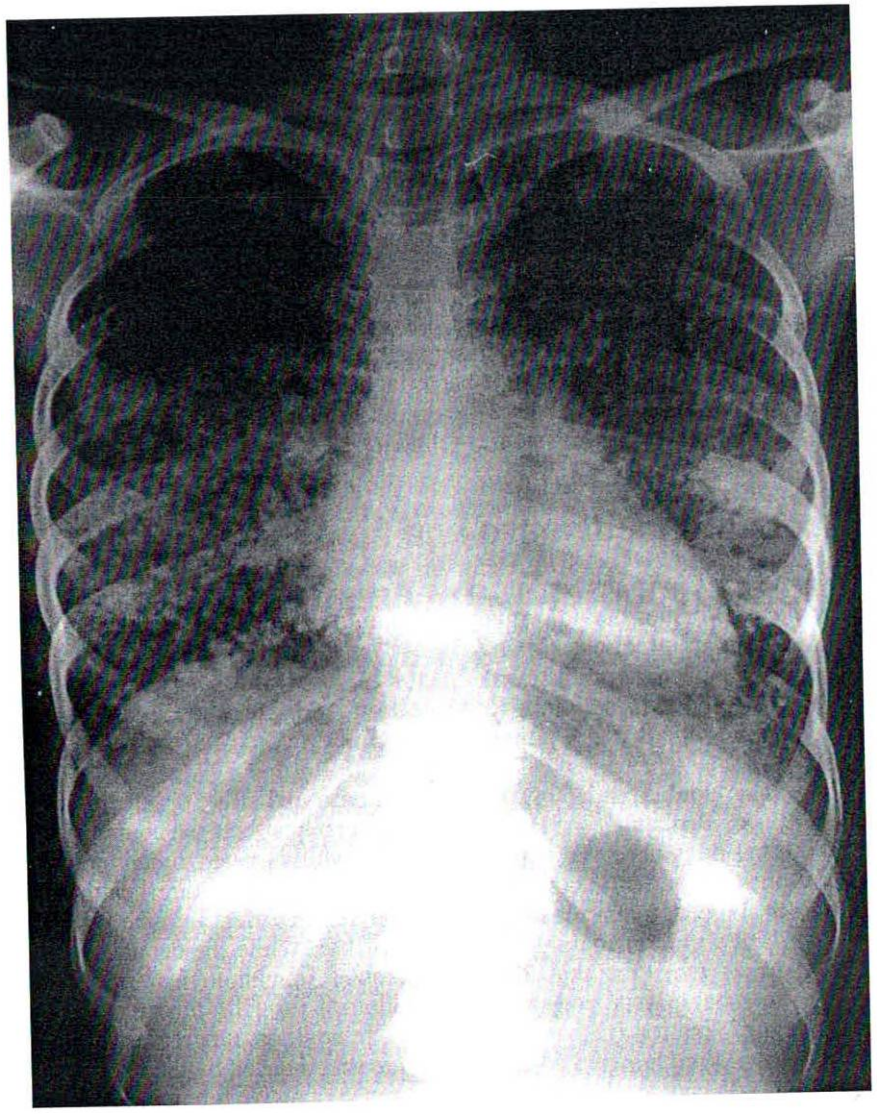

Fig. 5.12 Pulmonary tuberculosis. Diffuse X-ray changes with nodular lesions in all zones and no cavitation. These changes are often seen in patients with a poor immune response to *M. tuberculosis*. Courtesy of Dr P. Ormerod

lesions, as widespread lymphadenopathy or as multi-organ involvement. The latter differs from miliary tuberculosis as the discrete granulomas do not develop. Instead, organs contain minute necrotic foci teeming with acid-fast bacilli. These may not be visible radiologically and are only detected by biopsy or at autopsy. Thus, this form of the disease has been termed 'cryptic disseminated tuberculosis' (Proudfoot, 1971).

In contrast to tuberculosis, evidence for an interaction between HIV and leprosy is limited and conflicting. In general, clinical leprosy is no more frequent in HIV-positive than in HIV-negative individuals and, among leprosy patients, there is no relation between HIV status and the bacterial load. It has been observed that certain infections, notably those due to parasites and worms, are not adversely affected by HIV infection (Lucas, 1990), and it has been postulated that leprosy may be among these 'missing infections' (Lucas, 1993).

Summary – an integrated view of mycobacterial immunity

The host's mechanism for defence against mycobacterial disease resembles an orchestra with some lead performers such as the antigen-specific lymphocytes and macrophages and others with minor roles. In the past, the principal defence mechanism was thought to be the activated macrophage *per se* but attempts to demonstrate effective killing of *M. tuberculosis* by human macrophages have been elusive. It appears much more likely that effective defence requires the aggregation of many macrophages, thereby forming a granuloma. Although individual macrophages within the granuloma may well inhibit bacterial growth and kill some, the very hostile anoxic and acidic environment in the centre of the lesion may be much more important in overcoming the infection. In this respect, there are several immune mechanisms for seeking mycobacteria-laden macrophages and other cells and lysing them. These include natural killer cells, γ-δ cells and antigen-specific cytotoxic CD8+ (and some CD4+) cells. The targets for such lysis may be specific mycobacterial antigen or endogenous heat-shock proteins presented on the surfaces of stressed cells. In addition, healing processes surround the lesions with dense scar tissue, thereby enhancing the hostility of the internal environment to any residual mycobacteria and eventually walling off the resolving foci of disease.

Such immune mechanisms are usually very effective, leading to resolution of the initial lesions of tuberculosis and leprosy in about 95 per cent of cases. Unfortunately they seem unable to prevent the mycobacteria from entering the poorly understood 'persistor' state which may be responsible for post-primary disease in a minority of infected individuals years or decades later.

The protective immune responses are initiated by cytokines produced by TH1 T-cells, notably IFN-γ although TNF-α is essential for granuloma formation and subsequent fibrosis. Post-primary tuberculosis, which occurs in about 5 per cent of infected individuals, is associated with a mixed TH1/TH2 T-cell response which has the effect of rendering cells exquisitely susceptible to killing by TNF-α. This results in excessive tissue-necrotizing hypersensitivity which generates the cavities, without which transmission of the disease would not occur. Another feature of active tuberculosis is the suppressed immune recognition of common mycobacterial epitopes including those on heat-shock proteins. The exact significance of this 'immunological blindness' to protection is not clear but it could inhibit or delay the recognition and lysis of stressed cells laden with replicating bacilli.

The spectrum in leprosy is likewise related, at least in part, to the type of T-cell, TH1 or TH2. In tuberculoid leprosy the TH1 response causes formation of granulomas in nerves, thereby damaging them, and much of this reactivity may be aimed at bacterial debris that is not easily cleared form the tissues. In lepromatous leprosy the mixed TH1/TH2 response does not give rise to massive tissue necrosis as it does in tuberculosis because of a high degree of antigen-specific immune suppression mediated by CD8+ T-cells. A breakdown of this suppression appears to be responsible for leprosy reactions.

Many other factors are involved in immune responses to mycobacteria: the interactions of cells and cytokines are exceedingly complex. Nevertheless, there is increasing evidence that the 'choice' between health and progression to post-primary tuberculosis or multibacillary leprosy is related to the maturation pathway of the T-cells. This choice may be affected by genetic and, probably more critically, by environmental factors such as exposure to mycobacterial antigens that imprint the immune system with a pattern of subsequent responsiveness. There is evidence that this complex immunological orchestra is conducted by the endocrine system which determines the balance between hormones promoting TH1 and TH2 T-cell maturation pathways. There may indeed be a very complex interaction between antigen recognition, adjuvant activity, hormonal function and regulatory centres, such as the hypothalamus, in the brain. Whatever the nature and complexity of the regulatory system, the logical approach to the prevention and treatment of mycobacterial disease is to attempt to switch immune responsiveness from a mode that causes progressive disease to one that facilitates protection.

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4 Diagnostic mycobacteriology

Diagnostic mycobacteriology is a complex and technically demanding branch of medical microbiology. The organization of diagnostic services and the standard technical procedures have been reviewed in detail elsewhere (Collins *et al.*, 1985). The development of DNA-based diagnostic techniques such as DNA probes and the polymerase chain reaction could revolutionize diagnostic mycobacteriology although at the time of writing there are problems of sensitivity, specificity, cross-contamination and cost. The aim of this chapter is to enable the clinician to make the most of the available services and to outline the principles of the newer technologies.

The examination of clinical specimens suspected of containing mycobacteria by standard methods consists of four steps:

1. microscopical examination;
2. isolation of mycobacteria in culture;
3. identification of the organism;
4. drug susceptibility testing (where relevant).

In addition, reference laboratories play an important role in the collection of epidemiological information, staff training, quality control and research as well as giving clinical and technical advice. In many countries, laboratories are arranged in a hierarchical fashion. Microscopy and culture are performed in peripheral laboratories, larger centres identify *Mycobacterium tuberculosis* while the identification of other species as well as drug susceptibility testing are usually performed in designated reference centres. In New York State, USA, an additional component to the laboratory service is the 'fast-track programme' which uses modern technology and focuses on potentially highly infectious patients (Salfinger and Pfyffer, 1994). In poorer countries, emphasis is placed on microscopy services so that open or infectious cases of tuberculosis are detected and treated.

Laboratory safety

The handling of specimens and cultures exposes laboratory staff to a serious risk of infection. Workers in autopsy rooms are at particular risk. Safety measures are essential and, in some countries, mandatory. These include containment laboratories, approved safety cabinets and centrifuges, protective clothing, hand basins and facilities for the safe disposal of contaminated waste (World Health Organization, 1993). All staff should be vaccinated with the Bacille Calmette–Guérin (BCG) vaccine, unless they are tuberculin positive

or have a BCG scar, and must have medical supervision as determined by local policies.

Health and safety measures are no substitute for adequate training of staff in the basic technical procedures, their constant awareness of potential hazards and their familiarization with local safety rules. For a full account of laboratory safety see Collins (1993), Collins *et al.* (1995) and World Health Organization (1993).

Clinicians have a duty to consider the welfare of the laboratory staff by submitting specimens in a safe condition and by ensuring that specimen containers are not contaminated on the outside. Laboratory staff must be informed if the specimen comes from a patient known to be infected with a dangerous pathogen such as hepatitis B or the human immunodeficiency virus (HIV). Plastic bags containing tracheal catheters, bronchoscope traps and other objects contaminated with sputum are particularly hazardous and should be disposed of immediately without being opened. Request forms should never be placed in the same bag as the specimen. Plastic bags with separate pockets for the request forms are available.

The collection of specimens

The first, and most important, steps in any microbiological investigation are the collection and prompt delivery of suitable specimens. Much valuable information is lost, and time is wasted, by the submission of inadequate or mislabelled specimens or by delays in their delivery to the laboratory.

It is particularly important to collect all specimens into sterile containers. It is a common misassumption that, as specimens for mycobacterial investigations are 'decontaminated' before culture, the cleanliness of the container is less important than in the case of other bacteriological examinations. Unfortunately, unsterilized containers may be contaminated with environmental mycobacteria. There have been a number of 'pseudoepidemics' of apparent disease due to environmental mycobacteria as a result of collecting sputum or urine in clean but unsterile containers (Collins *et al.*, 1984). *Mycobacterium xenopi* is a common culprit as, being thermophilic, it survives in hospital hot water systems and contaminates sputum pots that are washed under the hot tap. Equipment used to collect specimens may likewise be contaminated: similar pseudoepidemics have been traced to the use of inadequately sterilized endoscopes (Gubler *et al.*, 1992).

Specimens

Sputum. The most suitable containers for sputum are wide-mouthed, screw-capped disposable plastic pots, into which sputum may be directly expectorated.

Most sputum specimens submitted for examination are 'spot' samples taken at clinics, but it is preferable to obtain at least three early-morning specimens. Sputum specimens are inevitably contaminated with non-acid-fast organisms which multiply rapidly. Specimens should therefore be

conveyed to the laboratory as quickly as possible. If delays in transport are unavoidable, sputum may be stored for up to one week at 0°C. If specimens are to be transported for long distances in hot climates and (as is often the case) refrigerated transport is not available, sputum may be preserved by the addition of an equal volume of 1 per cent cetyl pyridinium chloride in 2 per cent w/v saline.

Other respiratory tract specimens. Sputum is by far the best material for the diagnosis of pulmonary mycobacterial disease and all efforts should be made to obtain a specimen. Laryngeal swabbing and gastric aspiration are relatively ineffectual means of obtaining material suitable for culture, as well as being unpleasant for the patient. A more satisfactory alternative, if the equipment is available, is the use of the fibreoptic bronchoscope to take biopsies of radiologically visible lesions, to sample material on the bronchial walls by means of a small extendable brush, or to rinse out sections of the bronchial tree with saline (broncho–alveolar lavage). After use, the bronchoscope is sterilized with a 2 per cent solution of glutaraldehyde.

Laryngeal swabs are specially prepared for the purpose: they consist of stiff wires bent at one end at an angle of 35° and tipped with cotton wool. Some laboratories issue swabs tipped with alginate wool which can subsequently be dissolved so as to liberate the mycobacteria. Swabbing should only be done by staff trained in the technique: blind swabbing of the pharynx is useless. The operator should wear a visor as the patients inevitably cough violently during the procedure.

Gastric aspiration is performed in the early morning before food or drink is taken. The patient is requested to cough and swallow several times before the stomach contents are aspirated through a nasogastric tube. The aspirate should be transported without delay to the laboratory or, if this is not possible, it should be neutralized with sodium hydroxide.

Urine. Three early-morning midstream specimens should be obtained in 28 ml glass or plastic ('Universal') containers. Some laboratories isolate mycobacteria by membrane filtration of larger quantities of urine, such as total early-morning specimens.

Other fluids. Cerebrospinal fluid (CSF) and pus should be placed directly in sterile containers and sent to the laboratory without delay. Examination of CSF is an emergency investigation and the laboratory staff should be contacted by the clinician. Specimens of pleural, pericardial and peritoneal fluids may contain enough fibrinogen to cause them to coagulate. This is preventable by adding sterile sodium citrate to the specimen. Alternatively, such fluids may be added directly to an equal quantity of a double-strength liquid medium such as Kirchner or Middlebrook broth.

Tissues. Tissue is much more suitable for culture than necrotic material or pus, probably because the latter contain free fatty acids that are toxic to mycobacteria. For this reason the largest of a group of excised lymph nodes may not be the best for culture as it may be the most necrotic. Biopsies should be examined histologically as well as bacteriologically. This requires two specimens – one preserved in formalin for histology and the other one fresh for bacteriology.

Blood and bone marrow. In view of the increasing frequency of HIV-related disseminated mycobacterial disease, it is often necessary to examine blood and bone marrow. Radiometric techniques ('Bactec': see below) are ideal for the examination of these materials which are inoculated directly into the medium according to the manufacturer's instructions. Alternative culture methods include inoculating blood or bone marrow directly into liquid media or centrifuging anticoagulated blood lysed with sodium deoxycholate and inoculating solid media with the centrifuged deposit.

Faeces. Another consequence of HIV-related mycobacterial disease is the need to culture faeces for mycobacteria. Samples of 1–2 g should be submitted in sterile containers. Faeces are unsuitable for the diagnosis of tuberculosis in HIV-seronegative patients.

Microscopy

As the isolation of *M. tuberculosis* and most other pathogenic mycobacteria by standard cultural methods takes several weeks, the use of microscopy to reach a preliminary diagnosis is of great importance. In addition, microscopy of sputum is of great value in the detection of open or infectious cases of tuberculosis. It is well established that if no acid-fast bacilli are seen in sputum on a standard, yet competently performed, examination, the patient is unlikely to be infectious. (This may not be the case with HIV-seropositive patients.) Thus the establishment of good sputum microscopy services is of prime importance in developing countries where the first priority in tuberculosis control is the detection and treatment of the open cases.

Sputum is examined either directly or after liquefaction and centrifugation (see page 66). Direct examination is performed by selecting a purulent-looking portion of sputum and spreading it thinly on a glass slide with a bacteriological loop or a wooden throat swab stick. If centrifuged deposits are examined, great care must be taken to make sure that the reagents and diluents are not contaminated with environmental mycobacteria in order to avoid false-positive smears. There is evidence that concentration of bacilli in sputum by use of the cytocentrifuge increases the sensitivity of microscopy to that of culture on solid media (Saceanu *et al.*, 1993; Fodor, 1995).

Smears are stained by the Ziehl–Neelsen (ZN) method, or by one of its various modifications. In the traditional ZN method heat-fixed slides are flooded with carbol fuchsin (a phenol/water solution of basic fuchsin) and heated until steam rises. Boiling must be avoided as this dislodges the smear from the slide. After five minutes the slide is washed under the tap and then flooded with a dilute mineral acid such as 3 per cent sulphuric or hydrochloric acid. Some workers use acidified alcohol rather than acid alone as this tends to give a cleaner film. It does not, contrary to popular belief, distinguish tubercle bacilli from other mycobacteria.

After five minutes the slide is washed again and a counterstain is applied. The counterstain may be green or blue, depending on the microscopist's preference: red–blue colour-blind workers may use a yellow stain such as picric acid. Various 'cold' ZN staining techniques have been introduced but are not

An alternative to the ZN method is staining with auramine or rhodamine (or both) for fluorescence microscopy (FM). Although the equipment is more expensive, FM is much less tiring to the technical staff. Smears are examined at a lower magnification, thereby increasing the chance of detecting low numbers of acid-fast bacilli. Some authorities consider that fluorescent staining is less specific than ZN staining and advise that all smears found to be positive by FM should be confirmed by the ZN method.

Biopsies may be homogenized by grinding with sand in a Griffith tube or examined histologically by use of a modified ZN staining procedure such as that used by the US Armed Forces Institute of Pathology (see Ridley, 1988). Urines, cerebrospinal fluids and other fluids are centrifuged and the deposits are stained.

Culture media

The tubercle bacillus was originally isolated on heat-coagulated serum. Nowadays the most popular media contain egg and are solidified by heating at 80°C for one hour, a process termed inspissation. The widely used Löwenstein–Jensen medium contains eggs, asparagine, glycerol and some mineral salts. Stonebrink's medium is very similar to the former but contains sodium pyruvate instead of glycerol and is therefore suitable for the isolation of bovine tubercle bacilli. Egg-based media usually contain a dye such as malachite green which, in addition to being inhibitory to certain contaminating bacteria, gives a better background colour against which colonies of mycobacteria are more clearly seen. The nutritional role of egg is uncertain. The yolk may provide lipid precursors but it is doubtful if the albumin protein is utilized. It is more likely that the role of the protein is to absorb free fatty acids which are toxic for mycobacteria, although they stimulate growth if present in small quantities.

There are a number of clear broths or agar-based solid media but these are used more for drug susceptibility testing and research work than for primary isolation of strains from clinical specimens. These include Middlebrook–Dubos 7H9 broth and 7H10 agar, Sauton's medium and Kirchner's broth. For details see Collins *et al.* (1985). Liquid media are used in the radiometric culture technique (see below). A commercially available system (Septichek AFB system, Becton Dickinson) combining a liquid medium and a solid medium on a slide, permits detection of mycobacterial growth more rapidly than by standard culture but not as rapidly as by radiometry (Salfinger *et al.*, 1990).

The above media support the growth of almost all mycobacteria (except *M. leprae* and a few other non-cultivable species) but a few species require special media. The use of pyruvate instead of glycerol for the isolation of bovine tubercle bacilli has already been mentioned. *Mycobacterium paratuberculosis* and some strains of *M. avium* require media supplemented with mycobactin (see page 26) for growth. Such media are also preferable to standard media for the isolation of *M. avium* from specimens likely to contain only very few bacilli. Media containing iron supplements (haem or ferric ammonium citrate) are required for the isolation of *M. haemophilum*.

Decontamination of specimens

Most specimens submitted for culture of mycobacteria contain other bacteria and possibly fungi which, if not destroyed, will rapidly overgrow the medium. It is therefore necessary to treat the specimens with reagents that selectively kill non-acid-fast organisms. Fortunately mycobacteria are relatively more resistant to acids, alkalis and certain disinfectants than other micro-organisms. The difference in resistance is a relative one and no agent can be relied upon to kill all other organisms without also killing a substantial number of mycobacteria. Thus a decontamination procedure that permits a good isolation rate of mycobacteria will also result in a minority of cultures becoming contaminated – a fact that must be accepted by clinicians.

The choice of decontaminating agent depends on the specimen. 'Soft' methods are suitable for fresh specimens and for those likely to contain few contaminants, while 'hard' methods are used for heavily contaminated material. Soft reagents include trisodium phosphate and benzalkonium chloride, while hard reagents include sodium hydroxide and oxalic acid. The latter is particularly useful for the decontamination of urine specimens that contain *Pseudomonas aeruginosa* which is relatively resistant to alkali. In the case of sputum a liquefying agent, such as N-acetyl L-cysteine (NALC) or sodium lauryl (dodecyl) sulphate (SDS), may be added to the decontaminating reagent to aid concentration of acid-fast bacilli by centrifugation.

A popular decontamination procedure is that of Petroff. Sputum is shaken with an equal volume of 4 per cent NaOH (or 2 per cent NaOH with 1 per cent NALC) for 30 minutes and then centrifuged. The deposit is neutralized with dilute hydrochloric acid or 14 per cent dipotassium phosphate (KH_2PO_4), using neutral red as an indicator.

The 'sputum swab' method, although inferior to techniques in which sputum is concentrated, is suitable for laboratories without centrifuges. A throat swab dipped into sputum is placed first into a test tube containing 5% oxalic acid and is then transferred to another tube containing phosphate buffer. The swab is then used to inoculate a slope of Löwenstein–Jensen medium.

An alternative to decontamination is the use of a medium containing a 'cocktail' of antimicrobial agents that kill organisms other than mycobacteria. One of the most widely used media is Middlebrook–Dubos 7H10 agar containing polymyxin, trimethoprim, carbenicillin and amphotericin B (Mitchison *et al.*, 1983). Fewer than 1 per cent of plates seeded with untreated sputum become contaminated and the colony count of tubercle bacilli is 2.4 times higher than that on drug-free plates seeded with sputum decontaminated by treatment with NaOH. An alternative antibiotic cocktail (PANTA), used particularly in radiometric systems, contains polymyxin, amphotericin, nalidixic acid, trimethoprim and azlocillin.

Other types of contaminated specimens are treated in the same way. In the case of faeces, 1 g is decontaminated by the NaOH method, neutralized and added to 5 ml of a broth containing one of the antibiotic cocktails described above. Specimens such as tissue biopsies, CSF, pleural, pericardial and peritoneal fluids are usually sterile and may be directly inoculated on the appro- [illegible] specimens are inoculated on blood agar overnight to check for the need for a decontamination procedure.

Incubation and reading the cultures

There is a considerable variation in the temperature range of growth of the mycobacterial species – a feature utilized for identification purposes. Most mycobacteria grow at 35–37°C, but three species associated with skin disease, namely *M. marinum*, *M. haemophilum* and *M. ulcerans*, grow at a lower temperature. Thus all material from skin lesions should be incubated at 33°C as well as at a higher temperature. *Mycobacterium chelonae* may fail to grow at 37°C on primary isolation but grows at 35°C. Mycobacteria are aerobic but their growth is enhanced by an atmosphere of 5–10 per cent CO_2 in air. As CO_2 incubators are costly to obtain and maintain, they are rarely used in clinical practice.

The slopes should be examined weekly for at least 8 weeks and preferably for up to 12 weeks as some species, such as *M. xenopi* and *M. malmoense*, may take this time to appear on primary isolation. An even longer incubation, up to 14 weeks, is required in veterinary practice for the isolation of *M. paratuberculosis*.

Contamination is usually indicated by a softening or discolouration of the media. Mycobacterial colonies can usually, with experience, be distinguished from those of contaminating bacteria but confirmation should always be made by Ziehl–Neelsen staining.

Animal inoculation for the isolation of mycobacteria

Guinea-pig inoculation was once a popular way of diagnosing tuberculosis but it is now obsolete. It has been clearly demonstrated that the use of this animal offers no advantages over *in vitro* culture (Pallen, 1987).

Radiometric methods

In view of the slow rate of growth of tubercle bacilli and most other pathogenic mycobacteria, attempts have been made to develop rapid methods for detecting mycobacteria in specimens by their growth or metabolism. The only such rapid method in routine clinical use is radiometry. This technique, originally introduced for the detection of bacterial growth in blood cultures, is based on the release of radioactive CO_2 from a labelled precursor by bacterial metabolism. The released gas is detected by periodic sampling of the air space over the medium: this is done automatically in a commercially available instrument (Bactec 460/TB, Becton Dickinson). The specimen is added to a bottle of broth containing [^{14}C]-palmitic acid and a cocktail of antimicrobial agents, such as PANTA (see above), to inhibit growth of any organism other than mycobacteria. Growth of mycobacteria may be detectable within two or

three days, although most studies show that the isolation rate is no higher than by standard culture. A few strains of *M. tuberculosis* grow on conventional solid media but not in the radiometric system so both culture methods should be used.

Radiometry is also used for rapid drug susceptibility testing by incorporating antituberculosis drugs in the media. Although it is undoubtedly more rapid than conventional techniques, it is more costly and this restricts its use in routine diagnostic services in many countries. For further details of radiometric methods, see Heifets (1986).

Identification of mycobacteria

By far the most frequently isolated mycobacterium in clinical practice is *M. tuberculosis*. The first step in identification is therefore to determine whether or not an isolate is of this species or the closely related *M. bovis* and *M. africanum*. This can be done in several ways; for example, the following properties together clearly distinguish these strains from all other mycobacteria: slow growth rate, no pigment produced in the light or dark, no growth at 25°C and no growth on egg media containing 500 mg/l of *p*-nitrobenzoic acid or *p*-nitro-α-acetylamino-β-propriophenone (NAP).

Some workers identify the human tubercle bacillus by its production of large quantities of niacin, detectable by a simple chemical test (Konno, 1956) or by the use of commercially available test strips (Difco). Reliance should not be placed on this test alone as, in addition to bovine strains, a few human strains are negative. Conversely, positive reactions are given by *M. simiae* and by a few strains of *M. avium* and the rapidly growing pathogen *M. chelonae*.

Nucleic acid probes for the rapid identification of the *M. tuberculosis* complex and, specifically, of *M. tuberculosis* are commercially available (see page 74).

Typing tubercle bacilli

The tubercle bacilli that cause human disease are *M. tuberculosis*, *M. bovis* and *M. africanum*. In addition BCG occasionally causes localized or even widespread disease and may need to be distinguished from the virulent types. The subdivision of the virulent tubercle bacilli is for epidemiological purposes only as the clinical management of all these types is identical, except that bovine strains are naturally resistant to the antituberculosis drug pyrazinamide. A widely used typing scheme for these organisms is described on page 43 and the technical methods have been published by the World Health Organization (Grange and Yates, 1995).

Identification of other mycobacterial species

There is no universally accepted protocol for the identification of mycobacteria other than the tubercle bacilli. Ideally each strain thought to be the cause of disease should be identified at the species level and, in some cases, at the subspecies level so as to increase our knowledge of the types of mycobacteria that cause disease, and to determine the most appropriate therapy. Often, though, facilities and finance prevent such a thorough investigation.

Some laboratories are in the fortunate position of being able to perform a wide range of tests on each isolate and possibly to use highly discriminatory test systems, including nucleic acid probes and ribotyping. Others must be content with using a few simple tests which will identify most species that are encountered in clinical practice. Marks (1976) divided clinical isolates into 15 groups according to growth at 25, 37, 42 and 45°C, pigment production, oxygen preference and hydrolysis of Tween 80. Additional simple and useful tests include detection of nitratase and arylsulphatase activity and reduction of tellurite. Glycosidase activity is also of value, especially α-L-fucosidase activity which is particularly strong in *M. marinum* and also distinguishes *M. szulgai* from other slowly growing scotochromogens. The use of these and other cultural and biochemical tests for the identification of most of the slowly growing mycobacteria is shown in Table 4.1.

Rapidly growing species – those that give a good growth on subculture from a small inoculum on Löwenstein–Jensen medium within seven days – may be identified by a range of enzymic activities and substrate utilizations. In this respect, utilization of citrate, ammonia production from allantoin and acid production from mannitol, inositol and xylose and are of particular value, as shown in Table 4.2. From the clinical point of view the important rapid growers are *M. chelonae* and *M. fortuitum* as, with very rare exceptions, these are the only human pathogens in this group. These are distinguished from other rapid growers by their lack of pigment, strong arylsulphatase activity and limited saccharolytic activity, and from each other by the nitratase test and other properties shown in Table 3.5.

More sophisticated identification tests include characterization of mycosides (see page 21) by lipid chromatography, immunodiffusion analysis of cytoplasmic antigens, protein electrophoresis, gas–liquid chromatography, pyrolysis mass spectroscopy and ribotyping (see page 29). Nucleic acid probes for rapid identification of some of the commoner mycobacteria isolated in clinical laboratories are commercially available (see page 74).

Drug susceptibility testing

As outlined later (see Chapter 10), drug-resistant mutants continuously arise at a low rate in any mycobacterial population. Any culture will therefore inevitably contain a few such mutants. The purpose of susceptibility testing is to determine whether the great majority of bacilli in the culture are sensitive to the antituberculosis drugs currently in use. In other words, susceptibility tests are designed to inform the clinician whether or not an isolate is as susceptible to a given drug as other known sensitive strains.

There are four major techniques for susceptibility testing: the resistance ratio method, the absolute concentration method, the proportion method and radiometry. Testing may be direct or indirect, i.e. performed on the original specimen or on a subculture respectively.

Table 4.1 Properties of the slowly growing mycobacteria

	Pigmentation	Growth at (°C) 20	25	33	42	44	Nitrate reductase	Arylsulphatase	Tween 80 hydrolysis	Tellurite reduction	α-L-fucosidase	Urease	Acid phosphatase
M. tuberculosis	N	–	–	+	–	–	+	–	–	–	–	–	–
M. bovis	N	–	–	+	–	–	–	–	–	–	–	–	–
M. kansasii	P	–	+	+	v	–	+	+	+	–	–	+	+
M. marinum	P	+	+	+	–	–	–	+	+	–	+	+	+
M. asiaticum	P	–	+	+	v	–	–	(+)	+	–	–	–	+
M. simiae	P/N	–	+	+	+	–	–	(+)	(+)	–	–	+	–
M. scrofulaceum	S	v	+	+	–	–	–	–	–	–	–	+	–
M. szulgai	S	–	+	+	–	–	+	+	+	–	+	+	–
M. gordonae	S	+	+	+	+	v	–	+	+	–	–	+	+
M. avium–intracellulare	N	v	+	+	–	–	–	–	–	+	–	–	–
M. malmoense	N	–	–	+	–	–	–	–	(+)	–	0	–	–
M. ulcerans	Ny	–	–	–	+	+	–	–	–	–	–	–	–
M. xenopi	Ny	–	+	+	–	–	–	+	–	–	–	–	–
M. haemophilum	N	0	+	+	–	–	–	0	–	–	0	–	+
M. terrae	N	v	+	+	–	–	+	+	+	–	0	–	+
M. triviale	N	v	+	+	–	–	+	+	+	–	0	–	+
M. nonchromogenicum	N	v	+	+	–	–	–	+	+	–	0	–	+

N = nonchromogen; P = photochromogen; S = scotochromogen; Ny = light lemon-yellow colour; + = positive reaction in >85 per cent of strains; – = negative reaction in >85 per cent of strains; (+) = weak or late reaction; v = variable growth; 0 = limited or no data

Table 4.2 Properties of some rapidly growing mycobacteria

	Pigment	Growth at 45°C	Growth after 4 hours at 60°C	Arylsulphatase (3 days)	Nitrate reductase	Citrate utilization	Allantoinase	Acid from mannitol	Acid from inositol	Acid from xylose
M. fortuitum type A	–	+	–	+	+	+	+	–	–	–
M. fortuitum type B	–	–	–	+	+	+	+	+	–	–
M. fortuitum type C	–	–	–	+	+	+	+	+	+	–
M. chelonae abscessus	–	–	–	+	–	–	–	–	–	–
M. chelonae chelonae	–	–	–	+	–	+	–	–	–	–
M. smegmatis	v	+	–	–	+	+	–	+	+	+
M. phlei	+	+	+	–	+	+	–	+	–	+
M. diernhoferi	–	–	–	–	+	+	–	+	+	+
M. gilvum	+	–	–	+	+	+	–	+	+	–
M. duvalii	+	–	–	–	+	–	–	+	–	–
M. flavescens	+	–	–	+	+	–	–	v	–	–
M. vaccae	+	–	–	–	v	v	+	+	+	+

+ = positive; – = negative; v = variable result

The resistance ratio is determined by inoculating standardized suspensions of the test strain and a number of known sensitive strains on to media containing doubling dilutions of the drug. After incubation, the endpoint for each strain (i.e. the slope with 20 or fewer colonies) is determined. Test strains are then compared with the average or 'modal' resistance of the set of known sensitive strains. If the endpoint of test and controls is equal, the strain has a resistance ratio of 1. As doubling dilutions of drugs are used, 1, 2 or 3 tube differences in the endpoints of test and control strains give resistance ratios of 2, 4 and 8, respectively. Strains with resistance ratios of 4 or more are reported as resistant. Examples are shown in Table 4.3.

The absolute concentration method is very similar to the resistance ratio method, being based on a titration of the test strain, along with adequate control strains, on slopes of media containing known quantities of the drug in doubling dilutions. The difference is that the results are expressed in the actual endpoint concentration of drug. In practice, the activity of the drug may be less than its concentration in the medium owing to its denaturation during medium preparation. Accordingly, this method has no real advantage over the resistance ratio method.

In the proportion method the number of colonies growing from a standard inoculum on a drug-containing medium is compared with the colony count from the same sized inoculum on a drug-free medium. A strain is considered resistant to a given concentration of drug if the number of colonies growing on the drug-containing medium is 1 per cent or more of the number growing on the drug-free medium.

The three non-radiometric methods give essentially similar results. The resistance ratio method is used in Great Britain while the proportion method is widely used in the USA. More recently, radiometry (see page 67) has proved to be a rapid and reliable method for determining susceptibility to the first-line drugs and its rapidity justifies its higher cost.

For details of the standard techniques for drug susceptibility testing see Collins *et al.* (1984, 1995) and Vareldzis *et al.* (1993) and for radiometric methods see Heifets (1991) and Heifets *et al.* (1993).

Pyrazinamide susceptibility tests pose a particular problem as this drug is only active in acid media and there is a narrow pH range, around 5.2, at which the drug is active and the bacilli are able to grow. A reliable technique was described by Marks (1964) and modified by Yates (see Collins *et al.*, 1985; Grange and Yates, 1995). Pyrazinamide-resistant strains lack the enzyme, pyrazinamidase, required for conversion of this drug to its active metabolite. Thus, detection of this enzyme activity may prove to be a simple alternative to formal susceptibility testing, but this has not been adequately investigated.

Table 4.3 Examples of the resistance ratio method

	Increasing drug concentration (tube number)						*Resistance ratio*
	1	2	3	4	5	6	
Modal resistance	C	D	O	O	O	O	–
Test strain no.							
1	C	C	O	O	O	O	1
2	C	D	O	O	O	O	1
3	C	C	D	O	O	O	2
4	C	C	C	D	O	O	4
5	C	C	D	+	O	O	4
6	C	C	C	C	C	O	8
7	C	C	C	D	+	O	8
8	C	C	C	C	D	+	8+

C = confluent growth; D = numerous discrete colonies; + = 20–100 colonies; O = <20 colonies

Laboratory reports

A report of the microscopic examination may be issued soon after receipt of the specimen. As tubercle bacilli cannot be distinguished from other mycobacteria on the basis of microscopy the initial report should merely state that acid-fast bacilli are present. Some laboratories also report the number of bacilli seen. A scale widely used in Europe for reporting smear microscopy is shown in Table 4.4.

Table 4.4 Sputum smear microscopy: a scheme for reporting the number of acid-fast bacilli seen in high power fields (Collins *et al.*, 1995)

Number of bacilli seen	*Report*
None in 300 fields	Negative
1–2 per 300 fields	( ) Repeat test
1–10 per 100 fields	+
1–10 per 10 fields	++
1–10 per field	+++
>10 per field	++++

Isolation of a mycobacterium in culture usually takes from two to five weeks and at that stage it should be possible to say with a high degree of certainty whether or not the isolate is a member of the *M. tuberculosis* complex. A firm identification of the strain, together with results of susceptibility testing, is available after a further three or four weeks. Thus susceptibility results are usually available about two months after submission of the specimen, unless direct testing or radiometric methods are used.

Laboratory methods in leprosy

As *M. leprae* cannot be cultured *in vitro*, routine investigations are limited to the microscopic demonstration of the bacilli in clinical specimens although the polymerase chain reaction (PCR) will become increasingly available for the rapid detection of this bacillus. The usual specimens for the microscopic diagnosis of leprosy are slit-skin smears and nasal scrapings which are examined for the presence of acid-fast bacilli. These diagnostic procedures

are described in Chapter 7. Leprosy bacilli may be propagated in the footpads of mice and, by incorporating drugs in the animals' food, susceptibility testing may be performed. This is a laborious procedure that is only carried out in a limited number of reference and research centres. Alternative techniques for determining drug susceptibility of *M. leprae* have been developed and are mostly based on the uptake of radio-labelled substrates (reviewed by Grange, 1991). Radiometric techniques (Bactec system) may also be used to determine *in vitro* drug susceptibility of *M. leprae* (Tomioka *et al.*, 1992) although large numbers of bacilli, 5×10^7 to 1×10^8, are required. The PCR has also been used to detect *M. leprae* in tissues and preliminary observations indicate that it is sensitive and specific (Jamal *et al.*, 1994). In addition, rifampicin resistance may be rapidly detected by single-strand conformational polymorphism (see page 30) of the PCR-amplified *rpoB* gene of *M. leprae*.

Laboratory methods based on DNA technology

The three techniques that are increasingly being used in diagnostic laboratories are PCR to detect mycobacterial DNA in clinical specimens, nucleic acid probes to identify cultures, and restriction fragment length polymorphism (RFLP) analysis (DNA fingerprinting) to compare strains for epidemiological purposes. The principles of these methods are described in Chapter 2.

The diagnosis of tuberculosis using PCR has been extensively investigated but it has not proved as sensitive or as specific as originally hoped. A blind comparison study by seven laboratories all experienced in molecular technology revealed a wide diversity in sensitivity and specificity (Noordhoek *et al.*, 1994) and serious doubts have been raised as to the usefulness of PCR, in the form available in 1995, as a diagnostic tool (Grosset and Mouton, 1995). Nevertheless, much research activity is being undertaken and innovative modifications of the PCR in commercially available kit form are becoming available. One useful innovation is the amplification of specific rRNA, of which there are about 2000 copies in each cell rather than just one (or up to 20 in the case of insertion sequences) of a specific DNA sequence in the genome. Such amplification may be achieved without the need for thermal cycling, and test kits, which are commercially available, detect mycobacteria in clinical specimens with a high degree of sensitivity and specificity (Bodmer *et al.*, 1994).

Nucleic acid probes with non-isotopic detection systems are available for the *M. tuberculosis* complex, *M. tuberculosis*, *M. avium*, *M. intracellulare*, the *M. avium* complex, *M. kansasii* and *M. gordonae* and doubtless others will become available. These can be used to identify cultures from conventional media and from radiometric vials (Salfinger and Pfyffer, 1994). The accuracy of these is very high but not absolute. Cultures of *M. celatum* and *M. terrae* have, for example, been misidentified as *M. tuberculosis*. Thus identification by use of probes must be followed up by conventional confirmatory tests.

Molecular techniques may eventually replace the time-consuming drug susceptibility tests. Techniques are available for detecting mutations responsible for rifampicin resistance in the PCR-amplified *rpoB* gene (see

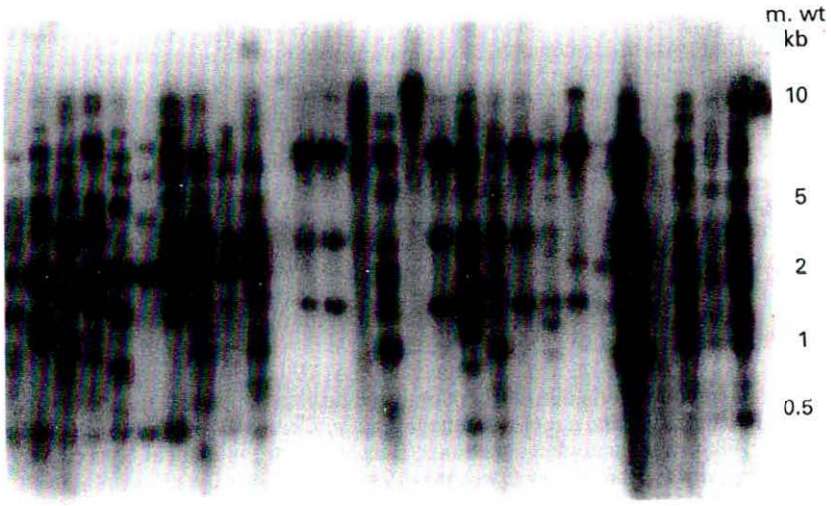

Fig. 4.1 *IS*6110 RFLP analysis of a collection of *M. tuberculosis* isolates (courtesy of M. Yates, Dulwich PHLS) including both classical European isolates and Asian strains. DNA was digested with PvuII, electrophoresed on 1% agarose gels, Southern blotted and hybridized with a ^{32}P labelled probe for *IS6110* and then autoradiographed. Some of the asian strains were not polymorphic, with only a few bands, resulting from low copy numbers of *IS6110*. Courtesy of Dr P. Butcher, St. George's Hospital, London

The technique of DNA fingerprinting has found several applications in epidemiological research (Stoker, 1994). It assists in detecting the source of infection, especially in 'explosive' HIV-related mini-epidemics and in distinguishing between exogenous reinfection and endogenous reactivation. It is also useful for detecting instances of laboratory cross-contamination of cultures. An example of the results of DNA fingerprinting is shown in Fig. 4.1.

Although such molecular techniques are not widely available, some countries have centres that perform PCR and DNA fingerprinting for specific purposes such as rapid diagnosis of tuberculous meningitis and investigations of outbreaks of disease. For further details on the clinical application of these newer techniques see Salfinger and Morris (1994) and Salfinger and Pfyffer (1994).

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10 Therapy of mycobacterial disease

The antimycobacterial agents

These are divisible into those substances that are only used for treating mycobacterial disease and those that have a much wider use. As a general rule, the modes of action of the drugs used specifically for treating mycobacterial disease are much less well understood than those of the agents also used for other purposes. For details of the pharmacokinetics and mode of action of the conventional antimycobacterial drugs see Winder (1982) and Bartmann (1988).

Tuberculosis

From time immemorial, the search for a cure for tuberculosis has been a major preoccupation of the medical profession. Indeed, it is salutary to recall that effective drugs have been available for only a few decades. Before the causative agent of tuberculosis was discovered in 1882, huge numbers of remedies were advocated: many were bizarre, most were unpleasant and virtually all were useless. Although Sir William Buchan's advice that the patient should suck woman's milk directly from the breast contrasts sharply in acceptability with John of Gaddeston's prescription of a mixture of pigeon's dung and weasel's blood, it was probably equally ineffective. Perhaps the only remedy of some value was cod liver oil, first used by Percival in 1770. This, owing to its high vitamin D content, may have exerted a beneficial effect in some cases of lupus vulgaris and other superficial tuberculous although it may have led to progression of pulmonary disease.

For about four decades after Koch's discovery of the tubercle bacillus, many workers, including Koch himself, attempted to develop immunotherapeutic regimens. Sphalinger (1922), for example, raised antisera in horses (Irish hunters) with which he treated a few patients, including President Nehru of India, with apparent success. The era of immunotherapy ended with the discovery of effective antituberculosis drugs, commencing with streptomycin by Waksman in 1948, but immunotherapy is once again set to play a major role in the control of tuberculosis (see page 220).

The importance of adequate chemotherapy in the control of tuberculosis has been well-established. Modern chemotherapeutic regimens are curative in the majority of cases provided that the tubercle bacilli are susceptible to the drugs and that a full course of therapy is taken. Ideally, treatment should be

themselves. For this reason, much research has gone into establishing the shortest duration of therapy and smallest number of doses that are compatible with a high cure rate.

Three other factors are of crucial importance to the success of chemotherapy as a component of tuberculosis control. First, effective drug regimens must be prescribed. A study in India showed that many private general practitioners prescribed inadequate and expensive courses of therapy (Uplekar and Shepard, 1991). Secondly, there must be a ready supply of drugs that are known to be effective. The bioavailability of component drugs in some commercially available combination tablets is suboptimal (Fox, 1990); some drugs are improperly stored or are time-expired and counterfeit drugs are marketed in some regions. Thirdly, patients should not have to pay for their drugs or supervision of therapy.

Susceptibility and resistance to antituberculosis agents

In the absence of mutational resistance, tubercle bacilli are remarkably uniform in their susceptibility to the antituberculosis drugs, although bovine strains are naturally resistant to pyrazinamide. Mutational change to resistance occurs at a low and constant rate (Table 10.1), which varies from drug to drug (David, 1970). Consequently, large populations of mycobacteria will inevitably contain a few bacilli that are resistant to a given drug. The purpose of drug susceptibility tests is not to detect these few mutants but to determine whether the great majority of the bacilli are susceptible. In the absence of the drug, the number of resistant mutants in a bacillary population remains low but in the presence of the drug the susceptible bacilli are killed and the resistant ones become dominant.

Principles of chemotherapy

The aim of therapy is to destroy all viable bacilli, rather than merely to reduce them to a very low level. This is not as straightforward as it might appear: drugs that are fully bactericidal *in vitro* may not achieve this effect *in vivo*. Mitchison (1985) has stressed the important difference between drugs that are bacteriostatic, those that are bactericidal under permissive conditions, and those that are capable of sterilizing lesions. Two further important properties

Table 10.1 Spontaneous mutation rate (per cell division) of *Mycobacterium tuberculosis* to rifampicin, isoniazid, ethambutol and streptomycin. Data from David (1970)

Drug	*Mutation rate*
Rifampicin	2.3×10^{-10}
Isoniazid	2.6×10^{-8}
Ethambutol	1.0×10^{-7}
Streptomycin	3.0×10^{-9}

Table 10.2 The ability of antituberculosis drugs to sterilize lesions, reduce viable bacterial population rapidly and prevent the emergence of drug resistance. Data from Mitchison (1985)

Drug	*Sterilizing*	*Early bactericidal*	*Prevention of drug resistance*
Rifampicin	Good	Fair	Good
Pyrazinamide	Good	Poor	Poor
Isoniazid	Fair	Good	Good
Ethambutol	Poor	Fair	Fair
Streptomycin	Poor	Poor	Fair
Thiacetazone	Poor	Poor	Poor

of drugs are their ability to prevent the emergence of resistance to a second drug, and their efficiency in destroying a large part of the bacterial population rapidly, thereby greatly reducing the infectivity of the patient (Table 10.2).

The mycobacterial population in a patient is functionally divisible into three groups:

1. freely dividing extracellular bacilli, mainly in the cavity walls;
2. slowly dividing bacilli within macrophages and in acidic, inflammatory lesions, and
3. dormant and near-dormant bacilli, within cells and in firm caseous material.

The most powerful sterilizing drug is rifampicin, being active against all three groups. Isoniazid is the most effective agent for destroying the freely multiplying extracellular bacilli, particularly those in the walls of cavities, but it is not a good sterilizing drug as it has limited efficacy against the metabolically less active intracellular bacilli which become relatively more frequent as treatment progresses.

Pyrazinamide is effective at a low pH, such as is found within macrophages and acidic, anoxic areas of inflammatory lesions, but it is ineffective against organisms at a neutral or high pH. Thus it is not good at preventing emergence of drug resistance as it only destroys part of the bacterial population.

Chemotherapy is divisible into three main phases. First, most of the freely multiplying extracellular organisms (group 1) are destroyed. It is in this phase, which lasts a week or two, that isoniazid has a major role. In the second phase, lasting perhaps a month or two, the remaining bacilli, principally within macrophages and inflammatory lesions, are killed by rifampicin and pyrazinamide. Finally, only dormant or near-dormant bacilli remain and are eventually killed by rifampicin. Despite the fact that isoniazid is usually only bactericidal during the early phase of therapy, it is used throughout the period of treatment as, by its bactericidal effect on replicating bacilli, it is very good at preventing the emergence of drug resistance.

Ethambutol has some bactericidal action in the early stage of therapy but it is not a sterilizing drug. Streptomycin is likewise not a sterilizing drug as it is bactericidal in alkaline areas of the cavity walls but is ineffective at the low pH

in some short-course regimens, notably those used when drug resistance to one or more of the other drugs is suspected or known or for retreatment of relapsing disease. Streptomycin has the disadvantage that it must be given by injection, which may lead to transmission of the hepatitis B virus and the human immunodeficiency virus (HIV) if needles and syringes are not sterilized.

Other antituberculosis drugs – ethionamide, prothionamide, thiacetazone, viomycin, cycloserine and *p*-amino salicylic acid (PAS) – are of much lower efficacy although some, notably thiacetazone, are still used as first-line drugs in certain countries on account of their low cost. These, and other, drugs are also used to treat multidrug-resistant tuberculosis (see below).

Design of chemotherapeutic regimens for pulmonary tuberculosis

Chemotherapeutic regimens are designed to cure the patient and prevent the emergence of drug resistance. In view of the problem of mutation to resistance, it is essential to give at least two drugs to which the strain is susceptible as the chance of two mutations occurring simultaneously in a single cell is negligible. In practice, particularly in regions with a high prevalence of drug resistance, this involves giving three or more drugs.

In the early days of antituberculosis therapy, it was considered necessary to give the drugs four times daily in order that inhibitory concentrations were constantly maintained. As therapy then lasted for up to two years, as many as 3000 doses were required. Subsequently it was shown that it was more effective to give the drugs daily and in some modern regimens they are given only thrice or twice weekly. Indeed, tuberculosis could be cured in the majority of cases with a mere 64 supervised doses.

For the reasons stated above, modern drug regimens are based on rifampicin and isoniazid throughout and one or two additional drugs during an initial intensive phase. The regimen currently recommended by the World Health Organization (1995) is known as Directly Observed Therapy, Short Course (DOTS) and consists of rifampicin, isoniazid, pyrazinamide and ethambutol daily for 2 months, and the first two drugs thrice weekly for a further 4 months. Each dose is taken under direct supervision by a competent supervisor.

A number of alternative 6-month regimens are in use; all are based on rifampicin, isoniazid and pyrazinamide with or without additional drugs and all consist of an early intensive phase and a continuation phase (Table 10.3; WHO, 1991; Centers for Disease Control, 1993). Some are daily and some are intermittent, although the latter should be replaced by the DOTS regimen outlined above whenever possible. Finally, although not recommended, 9- and 12-month regimens that do not contain rifampicin are still used in some regions.

The advantage of using an early intensive phase of treatment is that it probably cures a high proportion of patients so that there is less chance of the disease relapsing if the patient absconds before completion of the less-intensive continuation phase. The first- and second-line antituberculosis drugs and their recommended doses are listed in Table 10.4. For further details see Davies *et*

Table 10.3 Daily doses of antituberculosis drugs

Drug	*Dose* Adults	Children
Rifampicin	450 mg if body wt <50 kg 600 mg if body wt >50 kg	Up to 20 mg/kg to maximum of 600 mg
Isoniazid	200–300 mg	10 mg/kg
Pyrazinamide	1.5 g if body wt <50 kg 2.0 g if body wt >50 kg	35 mg/kg
Ethambutol*	15–25 mg/kg	As for adult
Streptomycin	750 mg if body wt <50 kg 750 mg if age >40 years 1 g if body wt >50 kg	20–30 mg/kg to maximum of 1 g
Thiacetazone	150 mg	4 mg/kg
PAS	8–12 g (divided doses)	200 mg/kg
Ethionamide	750 mg (divided doses)	15 mg/kg
Cycloserine	500 mg (divided doses)	Not recommended

* The 25 mg/kg dose should not be given for more than 2 months; additional therapy should be at 15 mg/kg. Ethambutol should only be given to children old enough to be tested for visual acuity (see under 'Drug toxicity')

Table 10.4 Some additional drug regimens for tuberculosis recommended by the World Health Organization (1991)

Intensive phase: 2 months	*Continuation phase: 4 months*
Standard regimen: daily throughout	
HRZ	HR
For use when resistance to one drug is suspected: daily throughout	
SHRZ	HR
EHRZ	HR
Intermittent regimens: thrice weekly throughout	
SHRZ	HRZ
EHRZ	HRZ

H = isoniazid; R = rifampicin; Z = pyrazinamide; S = streptomycin; E = ethambutol

Therapy of extrapulmonary tuberculosis

There is general agreement that the modern chemotherapeutic regimens discussed above are suitable for the treatment of all types of non-pulmonary tuberculosis, even life-threatening forms such as tuberculous meningitis. There is less agreement over the duration of therapy: many physicians continue therapy for up to 12 or even 18 months. There is no real evidence that this is necessary: indeed, limited trials suggest that treatment for the standard [illegible] The agents used in modern regimens penetrate tissues

well and the intrathecal administration of drugs in tuberculous meningitis is not necessary.

Surgery, now rarely used in pulmonary tuberculosis, is often of value, or essential, for the diagnosis and management of extrapulmonary disease. Examples include debridement or excision of bone lesions (with bone grafting if necessary), excision of non-functioning kidneys and repair of ureteric strictures, excision of lymph nodes and associated abscesses and sinuses, relief of intestinal obstruction due to abdominal disease, and relief of constrictive pericarditis.

Traditionally, tuberculosis of the spine was treated by radical surgery and bone grafting. Studies in several countries showed that, in the absence of neurological complications or marked deformity, radical surgery had no advantage over more simple debridement operations or even chemotherapy alone with bedrest or a plaster jacket if necessary (British Medical Research Council Working Party on Tuberculosis of the Spine, 1978). Other forms of bone and joint tuberculosis also respond well to chemotherapy and, in the absence of grossly destructive lesions or unstable joints, surgery is not required.

Therapy of multidrug-resistant tuberculosis

Multidrug resistance is an increasing problem in developing and developed countries alike. The occurrence and epidemiology is reviewed in Chapter 6. The generally accepted definition of a multidrug-resistant (MDR) tubercle bacillus is one that is resistant to rifampicin and isoniazid, as such resistance renders standard short-course therapy ineffective. Many MDR strains are also resistant to other drugs. In the USA, the Advisory Council for the Elimination of Tuberculosis (Centers for Disease Control, 1993) has recommended that, in populations or regions where MDR is common, drug susceptibility tests should be performed on all cases of culture positive tuberculosis; that patients should receive four drugs (rifampicin, isoniazid, pyrazinamide and either ethambutol or streptomycin) until the results of drug susceptibility tests are available and that all therapy should be given under direct observation by a responsible person.

Patients with MDR tuberculosis should be treated with at least three drugs selected on the basis of *in vitro* susceptibility. Unfortunately, such drug combinations are invariably less effective, more toxic and costly than the standard regimens. For maximum efficacy, treatment must be continued for 2 years after sputum cultures have become negative, adding enormously to problems of compliance. Even with prolonged and directly observed therapy, relapses often occur. In one large study (Goble *et al.*, 1993), there was a cure rate of 56 per cent except for HIV-positive patients in whom the mortality rate was 70–80 per cent. If therapy fails, the regimen should be revised in the light of the results of repeat *in vitro* susceptibility tests. Additional drugs should never be added blindly to an ineffective regimen.

Antituberculosis drugs suitable for treatment of MDR tuberculosis include ethionamide (or the closely-related drug prothionamide), rifabutin, amikacin, kanamycin, capreomycin, cycloserine and *p*-amino-salicylic acid (PAS). The

antileprosy drug clofazimine is one of a group of related compounds originally developed for treatment of tuberculosis and may again find application for treating this disease. The fluroquinolones, such as ciprofloxacin, ofloxacin and sparfloxacin and the new macrolides, clarithromycin and azithromycin, have been used with apparent success in a limited number of cases but controlled trials are required to establish their optimum use. As therapeutic regimens have to be 'tailored' for each patient on the basis of *in vitro* susceptibility, comparative studies on various regimens are not easy.

Most of the recent experience in the management of MDR has been obtained in the outbreaks in New York and other cities in the USA and is summarized by Dooley and Simone (1994), Morse (1994) and O'Brien (1994).

In view of the poor results of therapy of MDR tuberculosis, alternative forms of treatment such as immunotherapy (see below) are urgently required.

Treatment of patients with renal or hepatic disease

It is fortunate that rifampicin, isoniazid, pyrazinamide, ethionamide and prothionamide are either metabolized or eliminated in the bile. These may therefore be used safely at the normal doses in patients with renal impairment. Ethambutol is mainly eliminated by the kidney but can be used in reduced doses in patients with impaired renal function. Streptomycin and other aminoglycosides should be avoided as they are eliminated entirely by the kidney and, in addition, are nephrotoxic in some patients. Encephalopathy is an uncommon but serious complication of isoniazid therapy in patients with renal failure and those on dialysis but is preventable in most, but not all, cases by administering pyridoxine (Cheung *et al.*, 1993).

Patients with impaired liver function may be treated with isoniazid and ethambutol for one year, with the addition of streptomycin for the first two or three months. The more hepatotoxic drugs rifampicin and pyrazinamide are thus avoided, although in fact there is no evidence that these are any more toxic in patients with impaired hepatic function. It is important to monitor hepatic function regularly during therapy.

The role of adjunct steroid therapy in tuberculosis

Steroids have been used in the therapy of tuberculosis for two reasons. First, there was an assumption that, by reducing the host's immune response, the dormant bacilli would replicate freely and thereby become more susceptible to killing by the drugs. There is little evidence to support this conjecture and it has been shown that the use of steroids does not affect the outcome of modern short-course chemotherapy (Fox, 1978). Secondly, steroids are used to suppress damaging inflammatory reactions and subsequent scarring.

The use of steroids in tuberculous meningitis is controversial. Several controlled clinical trials have shown that they reduce mortality. They are certainly life-saving in the presence of cerebral oedema which sometimes develops after the commencement of drug therapy and is probably an allergic phenomenon. On the other hand, there is very little evidence that they reduce subsequent disability and it has even been stated that steroids merely delay death and prolong suffering (Escobar *et al.*, 1975). A small study suggested that dexamethasone reduced residual disability in both severe and mild/moderate cases but the authors stated that no statistically meaningful conclusions could be drawn and stressed the need for larger controlled studies (Kumarvelu *et al.*, 1994).

Prednisolone, with an initial dose of 60 mg and a gradual reduction over an 11-week period, reduces mortality, effusions and subsequent constrictive scarring in tuberculous pericarditis (Strang *et al.*, 1988). Steroids also reduce tuberculous pleural effusions but not subsequent formation of adhesions (Lee *et al.*, 1988). Other uses for steroids in tuberculosis include management of allergic drug reactions and gross enlargement of lymph nodes. Some clinicians give steroids in genito-urinary tuberculosis with the aim of preventing ureteric obstruction but the value of this therapy is unproven.

The use of adjunct steroid therapy in tuberculosis has been comprehensively reviewed by Alzeer and FitzGerald (1993).

Chemoprophylaxis and preventive therapy of tuberculosis

Chemoprophylaxis is defined as the prescription of antituberculosis drugs for uninfected people who are exposed to a risk of infection, while preventive therapy refers to the treatment of people who have already been infected with tubercle bacilli (as indicated by tuberculin testing) but show no clinical or radiological evidence of active disease.

These preventive measures are principally used to protect children who are at risk of infections, particularly those under the age of three years who are prone to develop serious extrapulmonary forms of tuberculosis, including meningitis.

The use of preventive therapy for adults, particularly those who are HIV-positive, is more controversial and is discussed in Chapter 6 (see page 132).

The role of preventive therapy in patients, other than transplant recipients (see below), who are receiving steroids is also controversial. It is often stated that, by compromising the immune response, steroids permit the reactivation of latent tuberculosis but the evidence for this is weak (Bateman, 1993) and neither short-term high-dose or long-term maintenance steroid therapy for obstructive airway disease reduced skin test reactivity or *in vitro* correlates of immunity to tuberculosis (Lowe *et al.*, 1987).

Transplant recipients receiving steroids and other immunosuppressive drugs are at risk of developing tuberculosis. It has been suggested that such patients should be given isoniazid, 300 mg, and pyridoxine, 25–50 mg, daily if they have one or more of the following: a history of inadequately treated tuberculosis, an abnormal chest X-ray, a positive tuberculin test of more than 10 mm in diameter and recent contact with a case of active tuberculosis (Qunibi *et al.*, 1990).

Supportive measures in the therapy of tuberculosis

This topic is discussed in Chapter 6 (see page 128).

Monitoring and follow-up of therapy

The purpose of monitoring drug therapy is three-fold: first, to ensure that the patient is taking the drugs; second, to determine whether the regimen is effective in the individual patient; and, third, to ensure that the drugs are having no harmful effects. The need to monitor patients during courses of modern short-course therapy by radiology and sputum culture is questionable and, in many regions, impossible. Likewise, the need for regular check-ups after the completion of therapy for fully drug-susceptible disease has been challenged. Even where such follow-up procedures have been applied (at considerable expense), almost all cases of reactivation were detected when the patient presented with a recurrence of symptoms (Albert *et al.*, 1976). Accordingly, resources should be used to ensure that every patient receives a full, supervised course of therapy rather than for any follow-up procedures (Rouillon *et al.*, 1976). Patients should then be informed that although they have almost certainly been cured they should seek medical advice promptly if symptoms recur. On the other hand, patients with multidrug-resistant tuberculosis require follow-up for at least two years after bacteriological cure.

Drug toxicity

Although all antituberculosis drugs have some untoward side-effects, drug toxicity is, in general, not a serious problem and is a small price to pay for the very real benefits of modern chemotherapy. The major side-effects are hepatotoxicity, peripheral neuropathy, mental disturbances, rashes and fevers. Side-effects are particularly likely to occur in HIV-positive patients.

The three principal drugs used in modern short-course regimens – isoniazid, rifampicin, pyrazinamide – are all potentially hepatotoxic but this is seldom a problem in clinical practice (Girling, 1989). The hepatotoxicity of pyrazinamide was over-emphasized in the past, when larger doses were given. It is generally recommended that liver function tests should be performed before commencing therapy but that they need not be routinely monitored during treatment unless the initial tests are abnormal or if the patient is an alcoholic or has liver disease. On the other hand, drug-induced acute liver failure, though rare, is extremely serious and some workers therefore advocate monthly liver function tests during therapy (Mitchell *et al.*, 1995).

Rifampicin may cause an influenza-like syndrome but, paradoxically, this is less likely to occur if the drug is given daily rather than twice or thrice weekly. Although the evidence that rifampicin is teratogenic is very limited, it is best avoided if possible during the first three months of pregnancy. For the same reason, women receiving rifampicin should avoid becoming pregnant. In this respect it is important to note that this drug interferes with the action of oral contraceptives. Alternative forms of birth control should therefore be used.

Isoniazid may cause peripheral neuritis and mild psychiatric disturbances which are usually preventable by giving pyridoxine (vitamin B_6) 10 mg daily. Although not prescribed routinely to all patients, pyridoxine should certainly be given to patients with liver disease, pregnant women, alcoholics, renal dialysis patients, HIV-positive patients, the malnourished and the elderly.

The rarely used second-line drug cycloserine also causes psychiatric symptoms, including hallucinations. Streptomycin is toxic for the eighth nerve including that of the foetus. For this reason, streptomycin should not be given during pregnancy.

Ethambutol has a very important side-effect; namely, ocular toxicity. Although this is rare if the drug is given for no more than two months at a daily dose of 25 mg/kg body weight, or for longer at a dose not exceeding 15 mg/kg, vigilance is indicated (particularly in litigation-conscious communities). A code of practice, suitable for use in developed countries with the requisite staff and facilities, has been recommended by the British Thoracic Society:

- Pre-treatment renal function should be investigated by assay of serum urea and/or creatinine; and ethambutol should not be given to patients with impaired renal function.
- The recommended dose of ethambutol and duration of therapy should never be exceeded.
- Any history of eye disease should be recorded in the notes.
- Pre-treatment visual acuity should be assessed by the Snellen test or, for those unable to read, by the Cambridge Low Contrast gratings. If the patient normally wears spectacles for distant vision they should be worn for the test. Ethambutol should not be given to patients with poor sight who may not notice further minor deterioration of vision.
- The small risk of ocular toxicity should be explained to the patient with an admonition to discontinue the drug if vision becomes impaired.
- A record should be made in the notes that the danger of ocular toxicity has been explained to the patient.
- The patient's general practitioner should be informed of the instructions given to the patient.
- Patients complaining of visual disturbance should be referred to an ophthalmologist.
- Routine tests of visual acuity during therapy are not recommended.
- Ethambutol should be avoided in patients who are unsuitable for objective tests of visual activity, i.e. young children and adults with language or other communication problems.

Thiacetazone is a common cause of rashes, particularly in patients of Chinese ethnic origin. More severe skin reactions, exfoliative dermatitis and the Stevens–Johnson syndrome, occur in less than 0.5 per cent of patients but there is a 10-fold increase in the incidence of these reactions in HIV-positive patients, proving fatal in up to 3 per cent of affected cases. Thus this drug should be avoided whenever possible and never given to a patient known to be HIV-positive.

Drug interactions

Clinically significant interactions between the first-line antituberculosis drugs themselves are uncommon but such reactions could well occur when more complex regimens are used to treat multidrug-resistant tuberculosis.

Antituberculosis drugs may interact with drugs used to treat unrelated conditions. Rifampicin is the most important in this respect as it is a potent inducer of cytochrome isoenzymes involved in the metabolism of many drugs. The drugs known to be metabolized by these isoenzymes include cyclosporin, oral contraceptives, corticosteroids, phenytoin, imidazole antifungals, theophylline and warfarin. The increased clearance of these drugs may lead to therapeutic failure unless levels are adjusted, and readjusted when rifampicin therapy ceases. Patients on oral contraceptives should be advised to use alternative forms of birth control (see above).

For a detailed review of antituberculosis drug interactions see Grange *et al.* (1994).

The role of drug susceptibility testing

The purpose of drug susceptibility testing, as outlined at the beginning of this chapter, is to determine whether the great majority of organisms in a culture are susceptible to levels of the drugs that are achieved clinically. Tests on pretreatment isolates will reveal whether the patient has been infected by a resistant strain, i.e. initial or primary resistance. Tests during therapy or following relapse will detect the emergence of resistant mutants, i.e. acquired or secondary resistance.

Susceptibility testing is indispensable in the assessment of new drug regimens and has epidemiological value in determining the pattern of resistance in a community. The need to determine and monitor the global incidence and distribution of drug resistance has increased recently due to the alarming reports of multidrug resistance in several countries, for which purpose the World Health Organization has proposed the establishment of a network of supra-regional reference laboratories (Nunn and Felten, 1994). The necessity or even desirability of testing every isolate in clinical practice is contestable. Susceptibility testing is expensive and time-consuming and requires a high level of technical expertise and rigid quality-control procedures. The test is not worth performing unless high standards of accuracy can be maintained. Much harm may be done by modifying a regimen to include less effective and more toxic drugs on the basis of a false report of resistance. Many more therapeutic failures result from irregular medication due to poor supervision or an erratic supply of drugs than to primary resistance (Grosset, 1978). On the other hand, where facilities are available, and where multidrug-resistant tuberculosis is common, such as New York, susceptibility testing of all clinical isolates is definitely indicated (Centers for Disease Control, 1993). The technical procedures for susceptibility testing are described in Chapter 4.

Leprosy

Principles of chemotherapy

The aims of the chemotherapy of leprosy are similar to those of tuberculosis; namely, the destruction of all bacilli and the prevention of relapses and the emergence of drug resistance. As discussed in Chapter 7, the number of bacilli in patients varies greatly and is related to the position of the patients on the immunological spectrum. Patients at or near the lepromatous pole have an enormous bacillary load and are presumed to be the most infectious. Furthermore, as they have little or no immune reactivity, the bacilli can only be destroyed by effective drug regimens. As in the case of tuberculosis, some bacilli persist for long periods of time, even in the presence of the bactericidal drug rifampicin (Pattyn *et al.*, 1976).

The earliest effective drug for leprosy was chaulmoogra oil, derived from the fruit of *Hydnocarpus wightiana*. This drug, the origin of which has inspired various legends, is of some efficacy in tuberculoid leprosy and may act by stimulating the immune responses. In 1943 Faget and his colleagues introduced promin which, although effective, was rather toxic and had to be given by intravenous injection. The same workers subsequently isolated the active principle of promin – diaminodiphenyl sulphone (DDS, dapsone) – which was cheap, very effective, virtually non-toxic and could be taken by mouth. In view of these properties, dapsone became the mainstay of antileprosy therapy, even though it is bacteriostatic rather than bactericidal. Side-effects are uncommon with dapsone, although cases of haemolytic anaemia and other blood disorders, hepatitis, dermatitis and psychosis occasionally occur. Agranulocytosis is a rare but serious complication. The term 'dapsone syndrome' is applied to a skin rash and fever, usually occurring 2–8 weeks after starting therapy and sometimes accompanied by lymphadenopathy, hepatomegaly, jaundice and/or mononucleosis.

The two other first-line drugs are clofazimine (B663, lamprene) and rifampicin. The former is weakly bactericidal; antibacterial activity against *M. leprae* is only demonstrable in human beings after 50 days of therapy. Clofazimine-resistant leprosy, though reported, is rare. It has a very long half-life, 70 days, and is eliminated in the urine and faeces. It is also anti-inflammatory and suppresses erythema nodosum leprosum reactions (see page 154). It occasionally causes gastrointestinal disturbances, relieved to some extent by taking the drug with a meal or glass of milk. It has the more serious disadvantage of causing skin discolouration which many patients find unacceptable. Discolouration of the hair, cornea, urine, sweat and tears also occurs. Infants born to mothers receiving clofazimine are reversibly pigmented at birth.

Rifampicin is bactericidal and causes a rapid killing of bacilli with a concomitant rapid reduction in infectivity. Indeed, about 99.9 per cent of bacilli are killed within a week of administering a single dose of 600–1500 mg of rifampicin. Unfortunately a minority of bacilli 'persist' and small numbers of viable drug-susceptible bacilli have been isolated from lepromatous patients after five years of rifampicin therapy (Waters *et al.*, 1978). The mechanism for such persistence, as in the case of tuberculosis, is unknown.

Other drugs shown to be effective in small clinical studies are ofloxacin (but not ciprofloxacin), clarithromycin and minocycline. These are recommended for the treatment of patients who refuse to take clofazimine or for whom rifampicin is unsuitable owing to drug toxicity or known drug resistance (World Health Organization, 1994). The related weakly bactericidal drugs ethionamide and prothionamide have been used together with rifampicin to treat patients who refuse to take clofazimine (Freerksen and Rosenfeld, 1977) but in view of

hepatic toxicity and gastric irritation, and the availability of other drugs, their use is no longer recommended (World Health Organization, 1994).

Drug regimens for leprosy

Until around 1976, dapsone monotherapy was the standard treatment for all forms of leprosy. The emergence of dapsone resistance in multibacillary patients had been reported and, although the incidence appeared to be very low, the World Health Organization Expert Committee on Leprosy (1977) stressed the need for studies on multidrug therapy for proven dapsone-resistant cases. At that time there were disturbing reports from some regions suggesting that the incidence of such resistance was higher than previously suspected and that it was increasing. The World Health Organization therefore sponsored a number of studies which confirmed the seriousness of the problem (World Health Organization Scientific Working Group on the Chemotherapy of Leprosy, 1982). Such emergent, or secondary, drug resistance is confined to patients with multibacillary cases, as only they have large enough numbers of replicating bacilli to make it likely that a mutation will occur. On the other hand, when such bacilli are transmitted to other persons, any form of leprosy may develop. Such primary resistance, unfortunately, now occurs in several countries (Waters *et al.*, 1978).

In view of the increasing frequency of both primary and secondary dapsone resistance, multidrug therapy (MDT) is now considered essential for the treatment of all forms of leprosy. Different regimens for multibacillary and paucibacillary cases have been recommended by the World Health Organization (1982, 1994), and are now used routinely in most countries where leprosy occurs. These MDT regimens are summarized in Table 10.5.

For the purposes of deciding which multidrug regimen to use, patients with negative slit-skin smears are classified as paucibacillary (PB) while those positive at any site are classified as multibacillary (MB). Relapsing patients with previous MB disease treated with dapsone alone are classified as MB for the purpose of retreatment, even if all slit-skin smears are negative. In practice, many patients are classified as PB or MB on the basis of clinical features rather than on microscopical findings.

Patients with MB disease require therapy for two years. As there is a huge bacillary load, there is a high chance that mutation to rifampicin resistance will develop. It is therefore essential to use three drugs. Patients with PB disease are treatable by short course regimens as any persisters are almost certainly destroyed by the patients' immune defences. As the bacillary load is small, the chance of drug resistance developing by mutation is very low. Accordingly a third drug is not essential.

As in the case of tuberculosis, relapses occasionally occur, particularly in MB cases (Waters, 1995) but, when tested for drug susceptibility, the bacilli from such cases are usually fully susceptible. In addition, residual antigen may continue to induce nerve damage and leprosy reactions after therapy is complete (Shetty *et al.*, 1992).

Non-compliance is a recurring problem and thus there is still a need for shorter or intermittent regimens. The advantages and problems of standard multidrug therapy and the possibilities for improved therapy based on alternative drugs such as ofloxacin, minocycline and clarithromycin are discussed by Ji and Grosset (1990), Grosset (1994) and the World Health Organization (1994).

Table 10.5 WHO recommended chemotherapeutic regimens for leprosy (WHO, 1994)

Standard regimens		
Paucibacillary leprosy	Rifampicin	– 600 mg monthly, supervised
	Dapsone	– 100 mg daily, unsupervised
Multibacillary leprosy	Rifampicin	– 600 mg monthly, supervised
	Dapsone	– 100 mg daily, unsupervised
	Clofazimine	– 300 mg monthly, supervised AND 50 mg daily, unsupervised
Alternative regimens		
Rifampicin resistance or toxicity	For 6 months, daily, under supervision:	
	Clofazimine	– 50 mg, and TWO of
	Ofloxacin	– 400 mg
	Minocycline	– 100 mg
	Clarithromycin	– 500 mg
	For an additional 18 months, daily, under supervision:	
	Clofazimine	– 50 mg and ONE of
	Ofloxacin	– 400 mg
	Minocycline	– 100 mg
Refusal to take clofazimine	Replace clofazimine in either standard regimen with daily, supervised:	
	Ofloxacin	– 400 mg OR
	Minocycline	– 100 mg

Chemoprophylaxis of leprosy

Household contacts of leprosy patients should be examined for signs of the disease and, if none are found, they should be advised to seek medical attention if any signs develop. Dapsone has been given prophylactically with some success but, in view of the medical and administrative problems generated by prophylaxis programmes, as well as the problem of dapsone resistance, this control measure is no longer recommended (World Health Organization, 1994).

Drug therapy of leprosy reactions

This subject is discussed in Chapter 7 (see page 154).

Therapy of the mycobacterioses

The treatment of the mycobacterioses, or opportunist mycobacterial diseases, is complicated by the problems of diagnosis and the difficulty in distinguishing between true disease and colonization; by the frequent occurrence of

serious underlying immune defects or other predisposing conditions; by the natural resistance of many mycobacteria to the available antimicrobial agents; the variation of this natural resistance within species and by the lack of correlation between *in vitro* resistance and response to therapy *in vivo*. The problem has been compounded by the frequent occurrence of such disease in patients with acquired immune deficiency syndrome (AIDS).

From the point of view of therapy, the mycobacterioses are divisible into three main groups:

1. pulmonary (and occasionally solitary non-pulmonary) disease due to slowly growing mycobacteria in HIV-negative patients;
2. disease, usually disseminated and due to the *M. avium* complex, in HIV-positive patients;
3. disease due to the rapidly growing species *M. chelonae* and *M. fortuitum*.

Treatment of the two named diseases, Buruli ulcer and swimming-pool granuloma, is discussed in Chapter 9.

Not all mycobacterial lesions require chemotherapy. Localized lymphadenitis in children almost always responds to surgical excision which is usually undertaken for diagnostic purposes. It is important that the whole infected node is removed as simple incision and drainage often leads to sinus formation, scarring and delayed healing. Children should be followed up for a few years to ensure that the disease does not recur or spread. Localized post-traumatic or post-injection lesions also usually occur in otherwise healthy individuals and tend to resolve spontaneously. Nevertheless, healing may be prolonged and complicated by the presence of discharging sinuses. Small lesions may be cured by simple excision and larger ones by curettage.

Pulmonary disease due to slowly growing species

The usual organisms are the *M. avium* complex, *M. kansasii*, *M. xenopi* and *M. malmoense*. In view of *in vitro* resistance of the *M. avium* complex to most of the antituberculosis agents, it was considered that standard triple antituberculosis therapy would be of no value. Instead, regimens containing five or six empirically chosen drugs were administered for up to 3 years (Davidson, 1976). This regimen was undoubtedly successful in a high proportion of patients who completed the therapy. Unfortunately many patients developed adverse drug reactions and compliance was poor. It was subsequently found that a more acceptable triple regimen of rifampicin, isoniazid and ethambutol is as effective for uncomplicated cases, provided that therapy is continued with all three drugs for at least 18 months (Engbaek *et al.*, 1984). This regimen has also proved effective for the treatment of pulmonary disease due to other slowly growing species, including *M. kansasii* (Banks *et al.*, 1983), *M. xenopi* (Smith and Citron, 1983) and *M. malmoense* (Banks *et al.*, 1985).

The inclusion of ethambutol for the whole duration of therapy is essential

bacterial cell. The role of isoniazid is less certain: disease due to *M. kansasii* infection responds well to a 9-month regimen of rifampicin and ethambutol (British Thoracic Society, 1994).

Despite the efficacy of therapy, relapse is not uncommon, especially in the case of *M. xenopi*. Thus surgical intervention, though rarely resorted to nowadays in the case of uncomplicated pulmonary tuberculosis, should be seriously considered for localized pulmonary lesions due to other mycobacteria (Moran *et al.*, 1983).

Experience with other drugs and species is limited and anecdotal. Strains of *M. kansasii*, *M. xenopi*, *M. malmoense*, the *M. avium* complex and *M. scrofulaceum* are usually susceptible to clarithromycin and fluroquinolones *in vitro* and there is anecdotal evidence of their efficacy *in vivo*. Co-trimoxazole has also been used in the successful treatment of cases of disease due to *M. xenopi* (Grange, 1984).

For further details on the therapy of disease due to the slowly growing species see Heifets (1994) and Banks (1994).

Rapidly growing mycobacteria

There have been very few controlled clinical trials of therapeutic regimens for disease due to the rapidly growing mycobacteria *M. chelonae* and *M. fortuitum*. Most cases are treated on the basis of anecdotal experience or by trial and error. As the drugs are almost always used in combination, it is difficult to assess the contribution of each agent to the result. Furthermore, the optimum duration of therapy has not been established and must therefore be based on the clinical response in individual patients. Regimens containing antituberculosis drugs, as used for treating disease due to slow growing opportunistic pathogens, are ineffective against the rapid growers.

Erythromycin has been used successfully together with amikacin or gentamicin (Wolinsky, 1979) or with co-trimoxazole (Azadian *et al.*, 1981; Jackson *et al.*, 1981). It has been suggested that the sulphonamide component of co-trimoxazole acts by enhancing the ability of the phagocytic cells to kill the bacteria (Tice and Solomon, 1979). It therefore appears to be of benefit even if the organism is resistant to it *in vitro* (Jackson *et al.*, 1981).

Doxycycline has been used with amikacin to treat infections caused by *M. chelonae* and *M. fortuitum* (Dalovisio *et al.*, 1981) and it was used with erythromycin and co-trimoxazole in the successful treatment of a disseminated *M. chelonae* infection in a renal transplant recipient (Azadian *et al.*, 1981).

Some beta-lactam antibiotics are active against the rapidly growing pathogens. Of the many cephalosporins, cefoxitin and cefmetazole are the most active *in vitro* and the former was used successfully with amikacin in the treatment of sternal infections due to *M. chelonae* following cardiac surgery (Kuritsky *et al.*, 1983). Imipenem is active against *M. chelonae* and the fluroquinolones against *M. fortuitum* (Yew *et al.*, 1990) but clinical experience with these drugs is limited. For further details of drug susceptibilities of *M. chelonae* and *M. fortuitum* see Cynamon and Klemens (1991) and for a review of therapy see McFarland and Kuritzkes (1993).

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Disseminated disease

The majority of cases are caused by the *M. avium* complex in AIDS patients. The design of suitable chemotherapeutic regimens has been hampered by the difficulty in assessing the drug susceptibility of members of this complex. A number of regimens based on rifabutin and the antileprosy drug clofazimine had been described (Iseman *et al.*, 1985). Subsequently, the macrolides clarithromycin and azithromycin were found to be highly effective, and should be included in all regimens (Masur and the US Public Health Service Task Force on Prophylaxis and Therapy for *M. avium* Complex, 1993). Companion drugs, essential to prevent emergence of drug resistance should, if possible, be selected on the basis of *in vitro* susceptibility tests (Heifets, 1994).

Disseminated disease due to other species in HIV-positive patients and such disease in HIV-negative patients is uncommon and treatment is based on the results of *in vitro* drug susceptibility tests.

Immunotherapy of mycobacterial disease

In view of the problems of non-compliance with therapy and the emergence of drug resistance in many countries, alternative forms of therapy for tuberculosis are urgently needed. The most promising is immunotherapy. The immunological basis of such therapy and the evidence that the non-pathogenic rapidly growing species *M. vaccae* has the properties required of an immunotherapeutic agent are summarized in Chapter 5.

This form of immunotherapy is a useful adjunct to chemotherapy of fully drug-susceptible tuberculosis but more impressive results have been obtained in the treatment of drug-resistant disease. Experience in Iran showed that immunotherapy gave good results in multidrug-resistant tuberculosis of recent onset but was less effective in very chronic cases with gross pulmonary scarring. Nevertheless, a 40 per cent cure rate was obtained, compared with a 1 per cent cure rate previously obtained with the limited range of available drugs (Etemadi *et al.*, 1992). Immunotherapy is particularly effective in regions where chemotherapy is very suboptimal. In Kano, Nigeria, where most of the available drugs were time-expired or fake, the mortality was very substantially reduced (Onyebujoh *et al.*, 1995): 19 of 47 (40 per cent) who received chemotherapy alone died during the ensuing 10–14 months while none of 34 who received both chemotherapy and immunotherapy died. A study in the same region showed that immunotherapy had a very beneficial effect in HIV-related tuberculosis: all 8 HIV-positive patients who were given the immunotherapy were alive after 2 years while 8 of 9 patients receiving placebo were dead and the other patient could not be traced (Stanford *et al.*, 1993). For more details on the theoretical and practical aspects of immunotherapy see Stanford *et al.* (1994).

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Index

Preface to the First Edition

The genus *Mycobacterium*, despite great advances in medical science, continues to be a major cause of misery and suffering throughout the world. Leprosy and tuberculosis attack the human race with undiminished vigour while several other species of mycobacteria are emerging as important causes of life-threatening disease.

It was originally envisaged that this book should be a revised version of my previous work *Mycobacterial Diseases*, published by Edward Arnold in 1988. In practice, there have been so many major developments in the subject over the last eight years that the text has been almost entirely rewritten. In particular, the drug treatment of leprosy, tuberculosis and other mycobacterial disease has become much more rational and considerable advances have been made in immunology, facilitated by the introduction of monoclonal antibodies, cell cloning techniques and modern 'genetic engineering' procedures. In addition, there have been advances in the ecology, biochemistry, epidemiology and classification of the mycobacteria. Sadly, though, there is an increasing gap between the 'high-tech' researchers and those responsible for the basic care of the victims of mycobacterial disease. I hope this book will help to bridge that gap.

This monograph provides a review of the mycobacteria themselves, their place in the environment, the way in which they interact with the living host, the nature of the diseases they cause and the available means of diagnosing, preventing and curing such disease. It is intended for both undergraduate and postgraduate students seeking a general account of the mycobacteria and the diseases they cause, for the clinician wishing to understand the underlying mechanisms of the pathogenesis of the diseases, for the epidemiologist and health care administrator wishing to appreciate the nature and magnitude of the public health problems posed by the diseases, and for the microbiologist providing a clinical service. The potential researcher will find an account of the exciting developments in the science of mycobacteriology and, more importantly, will become aware of the many gaps in our present-day knowledge!

John M. Grange
London, 1988

Preface to the Second Edition

In 1988, when the First Edition of this book was published, few would have predicted the enormous surge in interest in mycobacterial diseases that started in the early 1990s and culminated in the declaration of tuberculosis as a global emergency by the World Health Organization in 1993. A principal reason for the current interest and concern is the profound impact that the HIV/AIDS pandemic has had on the incidence and nature of mycobacterial disease in both the developing and the developed world. It is clear that, unless radical new control measures are introduced, HIV-related tuberculosis will have a catastrophic effect on human health worldwide in the twenty-first century. As a result, many researchers are attempting to develop new diagnostic, preventive and therapeutic measures by the application of molecular technology. Others are concerned with the infrastructure of health care and of making the best use of available tools for disease control. Interest is not limited to tuberculosis; as a result of the HIV/AIDS pandemic, other mycobacterial diseases that were once regarded as little more than curiosities are also now of major and increasing importance.

The one mycobacterial disease that has declined in incidence is leprosy, thanks to the widespread use of multidrug therapy. Nevertheless, there is still a pressing need to continue with the provision of care for those with this disease, many of whom will suffer from its effects for decades to come even though they are bacteriologically cured. Tuberculosis has taught us the danger of losing interest in an infectious disease before it, and its consequences, are truly eradicated.

As a result of this upsurge of concern, many scientists and medical practitioners are currently taking a serious interest in the mycobacteria and the diseases that they cause. The purpose of this book, like that of the First Edition, is to serve as a general introduction to this growing discipline. I hope it will prove invaluable to those about to embark on journeys of basic scientific discovery as well as to those attempting to control the mycobacterial diseases, often under very difficult conditions.

John M. Grange
London, 1996