Mycobacteria and their world

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Contents

Introduction ...................................................................................... 3
Mycobacteria and the natural environment. ............................................................. 4
Investigational aspects . . . . . . .................................................................... 4
Skin-testing with tuberculins . .................................................................... 4
The antigens of mycobacteria .................................................................... 4
Leprosy and tuberculosis . . . . .................................................................... 5
Buruli ulcer—M. ulcerans disease. ............................................................. 6
Other mycobacterial diseases occurring in man .................................................. 6
The Immuno-pathological spectrum of mycobacterial infections . . ........................................... 6
The Bacillus of Calmette and Guerin (BCG) ......................................................... 6
Mycobacterium vaccae—an immunomodulator for prophylaxis and therapy ................................ 7
Mycobacterium vaccae in prophylaxis . ............................................................. 7
Mycobacterium vaccae in therapy for leprosy and tuberculosis . . . . . . .................................... 7
Unexpected findings with M. vaccae ............................................................. 8
Mycobacteria and cancer . . . . .................................................................... 8
Immune modulation and genera related to mycobacteria. .................................................. 8
Tsukamurella inchonensis ........................................................................... 8
Gordonia bronchialis. ........................................................................... 9
Conclusions. ...................................................................................... 9
References ...................................................................................... 9

Introduction

Mycobacteria are ubiquitous, occurring everywhere in the world, except perhaps the Polar Regions. In deserts they can be found under rocks and among the dried roots of vegetation. Their favoured environmental habitat is close to, and at the edges of, fresh water both flowing and static. Besides their natural habitat in the environment, a few of their hundred or so species have become strict or obligate pathogens, and a number of species are opportunist pathogens, normally living in the environment but with the capacity to invade animal tissues. Thus, mycobacteria have two worlds: one in the external environment and the other in animal tissues, where they live in complicated relationships with the immune system. It is their pathogenic species causing leprosy, tuberculosis and Buruli ulcer that have made the mycobacteria notorious. The environmental species, seldom causing disease, have the greatest effects on the majority of healthy people. They were probably at least part of the driving force leading to the development of cell-mediated immunity during evolution in all animals. Environmental contact regulates susceptibility to many conditions other than those of mycobacterial aetiology and offers an unexpected means of protection from, and treatment for, humanity’s most important diseases.
Their potentially beneficial effects are also exerted by the other aerobic genera of the Actinomycetales, the order of bacteria to which the mycobacteria belong.

**Mycobacteria and the natural environment**

Although mycobacteria, except for the strict pathogens, all live in the environment of soil and water, their distribution is dictated by physical and chemical conditions. There is evidence that temperature, moisture, ultra-violet light, pH and geography affect this. In deserts the predominant species are chiefly of pigmented organisms, scotochromogens or photochromogens, of the slow-growing sub-genus. Their yellow carotinoid pigments protect them from sunlight, the ultra-violet bands of sunlight being lethal to non-pigmented species.

Mycobacteria as a genus, by virtue of their thick cell walls, generally survive drying and the effects of strong acids and alkalis, enabling them to pass through animal gut and be detected in faeces and in city sewerage outlets. The great majority of tubercle bacilli which become extracellular in the natural degradation of pulmonary lesions are either aerosolised or swallowed, thus becoming widely distributed in soil where they survive for long periods if protected from ultra-violet light [1,2].

In fresh-water situations, fast-growing species exceed the slow-growers and there is further selection by pH, some species preferring acidic conditions and others alkaline conditions. For example, *Mycobacterium vaccae* amongst fast-growers and *M. ulcerans* amongst slow-growers are more common in neutral to acid conditions than they are in alkaline ones.

**Investigational aspects**

In the early 1970s in an attempt to isolate *M. ulcerans* from the Ugandan environment, an extensive geographical survey of cultivable environmental species was carried out [3].

There are three ways of assessing mycobacterial distribution in the environment:

1. Bacteriological culture of environmental samples.
2. Polymerase chain reaction (PCR) probing of environmental samples.
3. Differential skin-testing of the resident human population.

All of these have been valuable. Culture sounds simple, but the methods required to isolate organisms, generally more slow-growing than much of the other environmental bacterial flora, are time-consuming and not always effective. Nonetheless, culture of an organism, the identity of which can be confirmed by phenetic, antigenic or genetic analysis, is the only absolute proof of its presence in an environment. PCR is generally reserved for searches for particular species, such as *M. ulcerans* [4], and for non-cultivable species, such as the leprosy bacillus [5]. Easier and faster is multiple skin-testing, depending upon the detection of remembered cellular immune responses in those living in the environment under investigation [6,7].

**Skin-testing with tuberculins**

Skin-testing with tuberculins is carried out by intradermal injection of soluble reagents prepared from various mycobacterial species. Reactions are read as diameters of induration after 48–72 h. When dealing with reagents made from pathogens, qualitative differences in positive responses can also be distinguished [8]. Safe and minimally invasive, these reagents are no longer available under current drug-regulatory conditions. However, in former times, enormous amounts of information were obtained [6–12].

There are interpretational difficulties owing to interference by previous vaccination with BCG, and to individuals responding to different groups of mycobacterial antigens, allowing a categorisation of responders [13,14]. Nonetheless, through these tests the interaction between man and his mycobacterial environment became apparent.

**The antigens of mycobacteria**

Immunodiffusion analysis of sonicate preparations of mycobacterial species against high titre antibodies raised in rabbits gives rise to the following definitions of four antigenic groups [15,16] (see Fig. 1):

1. Group i antigens are shared between all mycobacterial species and some of them are shared by all bacteria, including mitochondria in animal cells [17].
2. Group ii antigens are limited to the slowly growing species.
3. Group iii antigens are shared by many fast-growing species.
4. Group iv antigens are species-specific and limited to single species, or to small groups of closely related species [18].

In relation to these groups of bacterial antigens are the different categories of skin-test responders [13,14]:

1. Category 1 responders react to group i antigens, responding positively to tuberculins prepared from any mycobacterial species.
2. Category 2 responders do not produce positive responses to any of the tuberculins at their standard test concentrations; 10–20% of individuals fall into each of categories 1 and 2.
3. Category 3 responders react positively to the species-specific antigens of the species encountered. This category consisting of around 70% of individuals can be used to determine which species are present in an environment.
4. Category 4 is a minor category of those reacting to the group ii antigens of slow-growers [14].

Analysis of the results from category 3 responders reveals the immunological significance of environmental contact,
the species present, their immunising capacity and their frequency.

By skin testing with combinations of tuberculins prepared from four different mycobacterial species (the quadruple skin-testing system), the complications in interpretation can be in part elucidated [6–12].

**Leprosy and tuberculosis**

These two mycobacterioses share many characteristics, yet they are also quite distinct. As pathogens, their world is the internal environment of animal tissues, with the complication of the immune response. They share an immunological spectrum [19], of which each disease covers a part. Both leprosy and tubercle bacilli (M. leprae and M. tuberculosis, respectively) are intracellular pathogens, becoming extra-cellular when bacilliferous lesions break down; both exhibit persistence, the continued presence of living bacilli quiescent in the tissues in the absence of clinical disease [20,21]. These persisters may precede or follow active disease and can activate whenever the immunological situation is suitable for them. It is estimated that three quarters of the world’s human population carry tubercle bacilli without ever having had the clinical disease. Skin testing with leprosin (soluble reagent from leprosy bacilli harvested from experimentally infected armadillos) suggests that many people in leprosy-endemic countries are infected with leprosy bacilli. When immunity is perturbed, as with human immunodeficiency virus (HIV) infection, endogenous activation of tuberculous persisters occurs and susceptibility to exogenous infection is increased. In both leprosy and tuberculosis the question remains open as to whether persisters are complete, cell-walled organisms, or cell-wall free [22–24].

Whereas infectious particles of tuberculosis are dried droplet nuclei of aerosols exhaled from the lungs, those of leprosy are from the nose. They are considered the major source of new infections, with fomites and environmental aerosols occasionally giving rise to disease. These latter are thought to be the source of many opportunistic pulmonary infections with water-borne, slow-growing species [25,26].

The site of primary infection in tuberculosis is generally the periphery of the lung, whereas that of leprosy is thought to be the nasal mucosa [27]. Both diseases are associated with derangement of cellular immunity, and antibodies probably have little part to play beyond the establishment of infection. Infective particles of tubercle bacilli are thought to be small clumps of fewer than five bacilli, larger clumps being caught in the mucus flow of the airways and transported to the gut, a site much less susceptible to infection [28]. The same principles are likely to be true for leprosy bacilli. Good evidence exists proving that healthy, close contacts of leprosy patients may temporarily carry leprosy bacilli in their nasal mucosa that can be released as potentially infective particles [29,30].
Buruli ulcer—M. ulcerans disease

A disease of perturbed cellular immunity following the immunopathological spectrum of the mycobacterioses (see below), Buruli ulcer is unlike leprosy and tuberculosis. It is almost never passed from person to person, but is rather caught from the environment [31]. Very slow growing, even when the inoculum is taken from grossly bacilliferous lesions, it has only very recently been cultured from the environment [32]. Nonetheless, much can be learned from careful epidemiology. Unlike leprosy and tuberculosis, where virulence and pathogenicity are thought to be direct immunoperturbing characteristics of the bacilli, M. ulcerans causes the ulcerative and necrotising characteristics of Buruli ulcer by its production of a toxin [33,34]. This toxin, called “mycolactone” [35], is the result of the bacilli themselves being infected with a transmissible plasmid [36]. Non-toxigenic strains of M. ulcerans can arise during prolonged artificial culture and can be recognised by their inability to cause disease when injected experimentally into mice—animals very susceptible to the toxin [37]. In the absence of the occurrence of clinical Buruli ulcer, the environmental distribution of non-toxigenic M. ulcerans is hard to determine.

An assessment of the role contact with mycobacteria may play in susceptibility to juvenile asthma was carried out in Crete [38], an island where clinical cases of Buruli ulcer have not been recorded. More than the expected number of children coming from a village close to a freshwater lake responded positively to Burulin—a tuberculoid made from cultured M. ulcerans [39], indicating the probable presence of non-toxigenic M. ulcerans in their surroundings.

Other mycobacterial diseases occurring in man

These are opportunist diseases in which the tissues of susceptible individuals are invaded from environmental sources. They include swimming-pool granuloma caused by M. marinum, mycobacterial injection abscesses [40], frequently caused by M. chelonae or M. fortuitum, and mycobacterial cervicofacial adenitis caused by a range of slow-growing species, including M. scrofulaceum, M. avium and M. malmoense [41]. Chronic lung disease, usually of the elderly, can be caused by M. avium, M. intracellulare, M. kansasi and M. xenopi [42].

The Immuno-pathological spectrum of mycobacterial infections

The concept of an immunopathological spectrum proposed for leprosy by Ridley and Jopling [19,43] has been very influential. The spectrum embraces a series of phases related to bacterial growth. Prior to the spectrum, there is a latent phase which may vary from weeks to years in length. There is then a phase of uncontrolled bacterial growth when lesions are bacilliferous or multi-bacillary and antibody levels are high. As cellular immune reaction increases and antibody levels fall, bacillary numbers are reduced and a tuberculoid state supervenes. From the hosts viewpoint there is a stage of immune suppression with rapid development of disease associated with little cellular response and a stage of slowly progressive or static disease as a specific cellular response supersedes. The worst tissue destruction is usually associated with the change from immune suppression to recognition. Leprosy patients tend to remain either in a multi-bacillary lepromatous state or in a paucibacillary tuberculoid state. Those with the borderline types of disease, where most tissue damage occurs, are associated with movement, up or down, from suppressed to expressed cellular immune response [43]. Tuberculosis fits less easily than does leprosy into the spectrum, but can generally be compared with the borderline states in leprosy according to the activity of tissue destruction. The most tuberculoid form of tuberculosis progresses very slowly with few visible bacilli and limited tissue destruction.

Patients developing M. ulcerans infection (Buruli ulcer) pass through a latent phase of several weeks, into the clinical phase when ulceration begins under the immunosuppressive effects of mycolactone [35] and bacilli are present in millions. For unknown reasons, perhaps owing to production of antibodies that neutralise the toxin, the disease changes from multibacillary and necrotising to paucibacillary, tuberculoid and fibrosing [44,45] (see Fig. 2).

The Bacillus of Calmette and Guerin (BCG)

Following Jenner’s observations on vaccination, the discovery and development of vaccines effective against a number of infections took place in the second half of the 19th century. Attempts were made in the 1870s to develop a vaccine for tuberculosis, and Robert Koch’s work in the 1880s led to real progress [46,47]. Calmette and Guerin developed a suspension of live, attenuated bacilli isolated from a calf believed to be infected with the bovine form of tubercle bacillus, which could protect children from active tuberculosis [48]. BCG is an enhancer of cellular immunity, as were the earlier, less well-known products. These include Spahlinger’s vaccine produced from living, but dormant, tubercle bacilli [49] and Friedman’s preparation of live M. chelonae [50]. BCG gives a variable degree of protection from tuberculosis, leprosy [51,52], Buruli ulcer [53], malignant melanoma [54] and probably other cancers. We can only guess at the contribution that the widespread application of BCG vaccination has had on the changing patterns of many other diseases. BCG demonstrates that there is an immune response other than that expressed in the Ridley-Jopling spectrum, and that its efficacy is not dependent upon responses to species-specific antigens but to primitive antigens common to all bacteria and to the stress proteins of animal tissues [55,56]. Once this essential point has been grasped, progress can be made towards the recognition and development of immunoprophylaxis and immunotherapy. BCG is the only survivor of the early 20th century preparations of live organisms [49,50], though Friedmann’s “Anningzochin” was claimed to be effective against asthma, psoriasis and some other non-mycobacterial diseases as well as tuberculosis. These are claims revisited in the late 20th century and today with the recognition of the immunomodulatory powers of M. vaccae, M. bovone and of selected species from other genera of the aerobic Actinomycetales.
Mycobacterium vaccae—an immunomodulator for prophylaxis and therapy

In a search for an explanation of why BCG was so effective against leprosy in Uganda, but had little effect in Burma, the influence of priming by environmental species was investigated and it was found that the fast-grower \textit{M}. \textit{vaccae} enhanced protective post-BCG immune responses in Uganda, and slow-growers including \textit{M}. \textit{scrofulaceum} blocked them in Burma [57]. In London this could be demonstrated in laboratory mice [58], and it was shown that priming with killed \textit{M}. \textit{vaccae} alone also enhanced protective immune responses. This discovery of the potential value of sensitisation to \textit{M}. \textit{vaccae} came in the 1970s at the time of a great explosion of knowledge about immunology. The old idea that protective immunity and tissue-damaging allergy were different presentations of cellular immunity demonstrated in the tuberculin response was proven. At first these were designated “Listeria-like” and “Koch-like” responses, respectively [59]. These dubious terms were replaced by Th1 and Th2 as different pathways of maturation of CD4+ cells were recognised [60].

\textit{Mycobacterium vaccae} in prophylaxis

Transferred to the children of leprosy patients in Bombay [61], Iran [62–65] and later Vietnam [66], it was shown that a combined vaccine of live BCG plus killed \textit{M}. \textit{vaccae} was about 50% more effective than BCG alone in the induction of correlates of protective immunity, and the effect of killed \textit{M}. \textit{vaccae} alone was little different from the combination. A recent study has shown the benefits of repeated doses in preventing tuberculosis in HIV-positive patients [67].

\textit{Mycobacterium vaccae} in therapy for leprosy and tuberculosis

The potential for \textit{M}. \textit{vaccae} as an immuno-therapeutic for leprosy was first investigated in Spain [68,69], Iran [64,70], India [71] and Vietnam, and for tuberculosis in London [72], the Gambia, Kuwait [73,74], Nigeria [75], Romania [76,77] and Vietnam [78]. Subsequent studies found a single injected dose to be inadequate in some places, especially in HIV-infected persons [79].

A series of studies of tuberculosis were carried out in Argentina, with single injections [80], three injections [81] or 10 oral doses [82]. The principles of this type of immunotherapy, affecting host immunity and not directly the bacilli themselves, means that it would be equally effective against drug-resistant bacilli, and this has proved to be the case [73,76,83–85]. This was well demonstrated in a study at Mashad in Iran. A combination of anti-bacterial chemotherapy plus immunotherapy with \textit{M}. \textit{vaccae} should lead to less development of drug resistance and to substantially shorter treatment regimens.

\textbf{Fig. 2} – The immuno-pathological spectrum of the mycobacterioses. The basis of the spectrum for leprosy described by Ridley and Jopling [19] is shown in the upper section of the figure. To the left of the clinical spectrum is the period of latent disease and to the right of it is the period of persistent disabilities. Patients with clinical leprosy, except for those with borderline presentations, do not shift from multibacillary, lepromatous to paucibacillary, tuberculoid forms, without the application of immunotherapy. Untreated lepromatous disease can be life-long, whereas tuberculoid disease can be self-limiting. The middle section shows the situation of pulmonary tuberculosis in relation to the leprosy spectrum. Active tuberculosis is more analogous to lepromatous to borderline leprosy, and only healed cases correspond with tuberculoid leprosy. The bottom section shows Buruli ulcer (\textit{M}. \textit{ulcerans} disease) in relation to the leprosy spectrum. Coming from the environment there is a latent phase of several weeks’ duration, following which the disease passes through a multibacillary phase, analogous to lepromatous leprosy, when rapid extension of ulceration occurs. This is followed by a very rapid reduction of bacilli with the onset of cellular immune recognition, finishing with extensive disabilities in many cases owing to massive fibrosis.
The laying down of fibrous tissue is associated with type 2 cytokine activity and reports of M. vaccae immunotherapy for pulmonary tuberculosis record a reduction in radiological opacities and improved closure of cavities [75,77,80].

**Unexpected findings with M. vaccae**

During the period of investigation of immunotherapy for mycobacterial disease, patients and their physicians were asked to note effects on concurrent conditions. The first to be reported and investigated was psoriasis in a leprosy patient in South India [86], and this was confirmed in a study from Argentina [87]. Following this observation other autoimmune and related conditions have been investigated. The first of these was Raynaud’s disease and blood flow in the fingers of leprosy patients [70], from which success with M. vaccae led to studies of myointimal hyperplasia following balloon damage to the carotid arteries of rats [88]. Experimental periodontal disease in rats also responded to treatment with M. vaccae [89].

There have been conflicting reports from various countries suggesting that BCG vaccination has an effect on bronchial asthma, but this has been difficult to confirm [38]. Studies with M. vaccae in asthma and bronchial allergy in hay fever showed that its use is drug sparing so far as steroids and salbutamol are concerned and that the effect is on the delayed part of the immune response of airways, rather than their immediate responses [90].

Patients treated with M. vaccae have reported a reduction in susceptibility to the common cold and an observation made during studies on the prevention of tuberculosis in HIV-positive patients in Africa recorded an interesting reduction in malaria [67], which still has to be followed up. Atopic dermatitis in children responded well to a single injected treatment with M. vaccae, providing it was prepared in borate buffer [91,92], though a small effect was found on chronic severe dermatitis in adults, using the phosphate-buffered preparation [93]. Borate buffer appears to result in a more stable product than does the phosphate buffer, and discussions with a glycobiologist disclosed that autoclaving in borate breaks down proteins to short amino acid chains suitable for T cell activation, and better preserves the bacterial sugars. A comparative study in Kuwait found that killing M. vaccae by autoclaving produces a more effective product than does exposure to Co60 [74].

Two important properties of the M. vaccae preparation are expressed in immuno-prophylaxis and immunotherapy. The first is the immuno-modulating adjuvant activity of the cell wall complex down-regulating Th2 mechanisms [64]. The other is the content of antigens cross-reactive with invading bacteria and stressed host tissue [56], explaining the very wide therapeutic activities of the preparation.

**Mycobacteria and cancer**

At one time mycobacteria were considered as possible causes of cancer, but this has never been substantiated. However, mycobacteria do have the capacity to prevent and treat cancer. During the 1970s attempts were made to use BCG vaccine as an immunotherapeutic [94]. Some benefits were claimed, but the only successful application of BCG is by instilling it into the bladder of patients with carcinoma in situ [95], and currently this is the treatment of choice. The problem with BCG is that it enhances the cellular immune response for which the patient is already primed and does not modulate from Th2 towards Th1 maturation of T cells, an essential step in effective immunotherapy for cancer [96]. An extensive survey has shown that past BCG vaccination reduces susceptibility to malignant melanoma by about 50% and increases the successful outcome to treatment also by 50% [54].

With the development of M. vaccae and its use in patients with various conditions, remarkable effects have been observed in those with cancers [97–99]. Direct studies were performed in which patients with a variety of different malignancies received repeated intradermal injections, and a significant improvement in quality of life [100], better tolerance of the side effects of chemotherapy [99] and increased survival were achieved [98]. More recently, a combination of injections and oral capsules containing the same dose of M. vaccae have been used. An extensive and comprehensive list of patients with many different kinds of cancer has been progressing for several years and has produced some striking results.

Another mycobacterial species, M. obuense closely related to M. vaccae [101], was found to have similar immunotherapeutic activity and is being developed by Immodulon Therapeutics Ltd. in London [102]. It is undergoing clinical trials in malignant melanoma, carcinoma of the head of the pancreas and bowel cancer with liver metastases.

**Immune modulation and genera related to mycobacteria**

Among the aerobic, near-mycobacterial genera, within the Actinomycetales, are some species with adjuvant activities and antigens very similar to those of M. vaccae, but with subtle differences. Some of these species have been investigated for therapeutic activities in human, veterinary and agricultural medicine. Made in the same way as the M. vaccae preparation, useful results have been obtained.

**Tsukamurella inchonensis**

In animal models, a preparation of this species has been particularly successful in preventing inflammation of the intima of arteries damaged with a balloon catheter [88] and for use in the prevention and treatment of spontaneous type 2 diabetes mellitus. It was found that M. vaccae and T. inchonensis were the most effective, followed by Gordonia bronchialis and with Rhodococcus coprophilus being the least effective. Both of these are models of important human diseases. In man, anecdotal cases of asthma and periodontal disease treated with oral T. inchonensis have shown benefits similar to those achieved with M. vaccae. A marked reduction in frequency of common colds has been observed in volunteers taking oral therapy with T. inchonensis and patients with recurrent cold sores owing to Herpes simplex virus infection have found that the sores are of much shorter duration, less frequent and less severe. Currently, T. inchonensis is being investigated for potential...
introduction into human medicine by a new company, Actinopharma Ltd. In veterinary medicine, T. inchonensis has been found to be effective in the treatment of “sweet-itch”, a seasonal dermal allergy of horses [103], for which there is no other effective treatment, and in “heaves”, an allergic respiratory Airways obstructive disease of equines.

Gordonia bronchialis
Rats treated with G. bronchialis or R. coprophilus, but not with T. inchonensis, and challenged with live Trypanosoma cruzi show significantly reduced parasitaemias and less chronic myocarditis [104]. Pregnant rats treated with G. bronchialis, but not R. coprophilus gave birth to offspring protected from subsequent challenge with T. cruzi [105]. Potentially, these are models of the consequences of Chagas’ disease in man, and may have relevance in other causes of myocardial disease. In dogs, a preparation of G. bronchialis has been particularly effective against allergy to flea-bites [106] and is likely to be developed as veterinary medicine. In agricultural medicine, G. bronchialis induces improved survival and weight gain in shrimp and fish hatchlings and is improving growth rate and colour and reducing skeletal abnormalities in koi carp. Treatment with the same organism has resulted in improved growth rates in pigs and cattle, which is now being further investigated.

Conclusions
Among the 100 or more species of mycobacteria, M. tuberculosis and M. leprae are important pathogens and amongst the remainder are a number of species capable of becoming opportunistic pathogens, including the toxin-producing species M. ulcerans. Within the environment, moisture, temperature, pH, exposure to ultra-violet light and geography all have significant influences on mycobacterial distribution. Their presence probably provides an evolutionary drive towards the development of the immune systems of all animals.

Sensitisation of human and animal populations with locally common species offers a method of detecting their presence and provides a means by which susceptibility or protection from mycobacterial and other diseases can be determined. Killed preparations of selected mycobacteria and of species from other genera within the order Actinomycetales are potent immune modulators and enhancers useful in the prevention and treatment of many diseases in which cellular immunity plays a large part. Between them they provide products likely to transform the face of modern medicine.

References


