An investigation of the killing of *Mycobacterium tuberculosis* by macrophages and the acid stress response of *Mycobacterium smegmatis*.

by

Lyn O'Brien

Department of Microbiology and Immunology

University of Leicester

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An Investigation of the Killing of Mycobacterium tuberculosis by macrophages and the acid stress response of Mycobacterium smegmatis.

Attempts were made to activate human monocytes with immunomodulators and human macrophages with T-cell supernatants for in vitro antimycobacterial activity. Both these approaches failed. Alveolar macrophages from Mycobacterium bovis BCG-vaccinated guinea pigs have previously been shown to kill Mycobacterium tuberculosis in vitro. The guinea pig was therefore used to investigate macrophage antimycobacterial mechanisms.

Tuberculocidal factors were found within lysosomes. Lysosomal fractions of macrophages from BCG-vaccinated guinea pigs significantly (P< 0.001) killed M. tuberculosis. Tuberculocidal activity was not observed with macrophage lysosomal fractions from non-vaccinated guinea pigs. The specific activities of enzymes in macrophage homogenates were tested. None of the lysosomal enzymes had significantly different activities in macrophages from vaccinated and non-vaccinated guinea pigs.

Strains of M. tuberculosis were sensitive to reactive nitrogen intermediates (RNI). However, guinea pig alveolar macrophages did not generate RNI on infection with mycobacteria. Macrophages from BCG-vaccinated guinea pigs killed M. tuberculosis in the presence of an inhibitor of RNI synthesis. Thus, no evidence was gained to suggest that RNI are responsible for the tuberculocidal activity of guinea pig macrophages.

Aminoaldehydes have been shown to be toxic to M. tuberculosis. Ornithine decarboxylase (ODC) is the first enzyme in the pathway that synthesize aminoaldehydes. ODC activity was not elevated in macrophages twenty-four hours and six days after BCG vaccination. Increased ODC activity was not observed in macrophages infected with M. bovis BCG for twenty-four hours. ODC is therefore not responsible for any prolonged increase in aminoaldehyde production that may take place when macrophages are infected with mycobacteria.

Mycobacterium tuberculosis was passaged once through the mouse. After passaging, the mycobacteria exhibited increased resistance to hydrogen peroxide but not RNI. This indicates that hydrogen peroxide is generated during the murine immune response to mycobacteria.

Culturing Mycobacterium smegmatis at pH5.0 allowed the bacteria to survive subsequent incubation at pH3.5 better than bacteria grown solely at pH7.5. Mycobacterium smegmatis could therefore be adapted to lethal pH values by pre-exposing the bacteria to mildly acidic conditions.
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ABBREVIATIONS

ADC: Albumin Dextrose Complex
AIDS: Acquired Immune Deficiency Syndrome
AMP: Adenosine Monophosphate
ATR: Acid Tolerance Response
BCG: Bacille Calmette Guerin
CFU: Colony Forming Units
CMI: Cell-mediated Immunity
CSF: Colony Stimulating Factor
DMFO: Difluoromethylornithine
DMSO: Dimethylsulfoxide
DNA: Deoxyribonucleic Acid
DTH: Delayed-type Hypersensitivity
EDTA: Ethylenediaminetetraacetic acid
ELISA: Enzyme Linked Immunosorbent Assay
GIF: Growth Inhibition Factor
GMCSF: Granulocyte-Macrophage Colony Stimulating Factor
HIFCS: Heat Inactivated Foetal Calf Serum
HINCS: Heat Inactivated Newborn Calf Serum
HIV: Human Immunodeficiency Virus
HBSS: Hanks Balanced Salt Solution
HBSS-Hepes: HBSS plus 2.5mM Hepes, pH7.4
HRP: Horse Radish Peroxidase
IDO: Indoleamine 2,3-dioxygenase
IFN-γ: Interferon-gamma
IL: Interleukin
iNOS: Inducible Nitric Oxide Synthase
LAM: Lipoarabinomannan
LPS: Lipopolysaccharide
MIC: Minimum Inhibitory Concentration
mRNA: Messenger Ribonucleic Acid
NADPH: Nicotinamide Adenine Dinucleotide Phosphate (Reduced Form)
N\textsuperscript{\textcircled{O}}MMA: N\textsuperscript{\textcircled{O}}-Monomethyl-L-Arginine
NK: Natural Killer
NOS: Nitric Oxide Synthase
OADC: Ovalbumin Dextrose Complex
ODC: Ornithine Decarboxylase
PAO: Polyamine Oxidase
POHPAA: p-Hydroxyphenyl Acetic Acid
PMA: Phorbol Myristate Acetate
PNS: Post-nuclear Supernatant
PPD: Purified Protein Derivative of tuberculin
RIV: Root Index of Virulence
RNI: Reactive Nitrogen Intermediates
SIN-1: 3-morpholinosydnonimine-hydrochloride
SVEH: 2.5M sucrose, 1mM EDTA, 0.1% (v/v) ethanol and 5U ml\textsuperscript{-1} heparin, pH7.4
TB: Tuberculosis
TGF: Transforming Growth Factor
TH\textsubscript{1}: T-helper-type-1
TH\textsubscript{2}: T-helper-type-2
VBC: Vaccinated, Boosted and Challenged
ZN: Ziehl Neelsen
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1. INTRODUCTION.
1.1. Historical Background.

Tuberculosis (TB) is an ancient human disease. Tubercle bacilli have been found in human remains dating back to 5000 B.C. (Sager et al., 1972) and in Egyptian mummies from around 3500 B.C. (Zimmermann, 1979). The contagious nature of tuberculosis was first recorded by Aristotle (384-322 B.C.) (Meachen, 1978). In 420 A.D., Vegetius remarked that animals were victims of tuberculosis as well as man (Evans, 1994). Indeed, it has been speculated that cattle were the initial source of human tuberculosis infection and that *Mycobacterium tuberculosis* was a mutant of *Mycobacterium bovis* (Rich, 1944; Manchester, 1984). *Mycobacterium bovis* has a broad host range, capable of infecting man and several other species, whereas *M. tuberculosis* is naturally pathogenic only to man (Rich, 1944).

Fracastorius (1483-1553) believed in the existence of imperceptible particles as the cause of the disease some three hundred years before the discovery of the bacillus (Webb, 1936). Desaulut (1675-1737) accurately postulated that the disease was spread by infected sputum. In 1865, Villemin transmitted tuberculosis from man to rabbit by inoculation of infected material (Piery and Roshem, 1931). Not long afterwards, in 1882, the famous discovery of the tubercle bacillus was announced (Koch, 1882). Robert Koch stained and identified *M. tuberculosis* with aniline dyes, developed a method to culture tubercle bacilli outside the body and inoculated bacteria into experimental animals producing tuberculous lesions. Such was the magnitude of Koch's discovery that, from 1882 onwards, the sputum of suspected TB cases was stained and analysed for tubercle bacilli (Evans, 1994).

Koch initially believed that there was no difference between human and bovine tubercle bacilli. However, in 1898, Theobald Smith demonstrated that there were microscopic, morphological and toxic differences between the two bacilli (Smith, 1898). Five varieties of tubercle bacilli have now been distinguished (Evans, 1994). These are human, bovine, avian, murine and piscine. The human bacillus is at present thought to account for 98% of pulmonary tuberculosis cases and 70% of non-pulmonary forms in man (Evans, 1994). Before Pasteurisation was introduced, the bovine bacillus was commonly acquired by drinking infected milk. Rarely, it was acquired by eating infected beef. The disease caused by the bovine bacillus is related to the non-pulmonary forms of tuberculosis, such as intestinal and abdominal
tuberculosis.

Over the centuries \textit{M. tuberculosis} has taken a massive toll on human life. Overcrowded cities, appalling living and working conditions and poor nutrition have all contributed to the spread of tuberculosis. In 1650, tuberculosis was a leading cause of death recorded in London's Bills of Mortality (Evans, 1994). It has been estimated that in Europe, in the early nineteenth century, 30% of all deaths under fifty years of age could have been attributed to tuberculosis (Evans, 1994). In the middle of the nineteenth century, TB was responsible for around 20% of all deaths in England (Dubos and Dubos, 1952). It was not until the twentieth century that two major advances in the fight against tuberculosis were made. These were vaccination and chemotherapy.

Calmette and Guerin, working in France in the 1920s, developed an attenuated strain of a tubercle bacillus of bovine origin (Bacille Calmette Guerin; BCG) by repeated subculture on potato-bile medium. It was found to be incapable of producing tuberculosis in laboratory animals (Calmette, 1923). French children were soon receiving an oral vaccine but initially BCG vaccination was not popular in the rest of Europe. Meanwhile, the Scandinavian countries were administering BCG intradermally and by 1950 this was being offered throughout Europe in a mass vaccination campaign to all children. However, BCG vaccination has never been established in the U.S.A.

Unfortunately, the efficacy of BCG vaccination in adults varies greatly from region to region (Bloom and Fine, 1994). For example, when adults were vaccinated with BCG the result was approximately 80% protection in the United Kingdom but only 30% protection in India (Bloom and Fine, 1994). Various explanations have been given for the regional variation. One possibility is that immunity to mycobacterial disease may be conferred by exposure to environmental mycobacteria and that subsequent BCG vaccination cannot add substantially to the level of protection (Cheng \textit{et al}, 1993). However, even with these drawbacks, BCG vaccination still remains the most cost-effective antituberculosis control measure available to many developing countries.

Waksman, working in America, discovered streptomycin in 1944 (Schatz and Waksman, 1944). He found that it was bacteriostatic towards \textit{M. tuberculosis} and successful clinical trials
were subsequently set up in the U.S.A. and in Britain. In Sweden, Lehmann detected tuberculostatic activity with para-aminosalicylic acid (Lehmann, 1946). The clinical trial that followed this discovery, established that a combination of the two drugs allowed therapy to be continued for prolonged periods without the development of drug resistance (Medical Research Council Investigation, 1950). Unfortunately, the side effects of para-aminosalicylic acid were severe and so the discovery of a new drug, isonicotinic acid hydrazide (isoniazid), by Robitzek and Selikoff in 1952 was welcomed (Robitzek and Selikoff, 1952). Clinical trials demonstrated that bacterial resistance developed when isoniazid was used alone (Medical Research Council Investigation, 1952) but in combination with streptomycin it was shown to be the most effective remedy available (Medical Research Council Investigation, 1955). Since then, a number of new drugs have been discovered, including pyrazinamide, ethambutol and rifampicin. The introduction of chemotherapy brought about a sharp decline in the incidence of tuberculosis, especially in developed countries which could afford to instigate multi-drug treatments. At this time, optimism was high that tuberculosis could finally be eradicated after centuries of causing disease.

1.2. The Microbiology of Mycobacteria.

Mycobacteria are slow-growing, rod-shaped organisms, typically measuring 0.4x3μm, that are strictly aerobic, non-spore forming and non-motile. When growing in liquid media, the bacilli form clumps due to the hydrophobic nature of the cell surface (Wheeler and Ratledge, 1988). The cell wall of mycobacteria is unique. It is composed of an interlacing layer of lipids, peptidoglycans and arabinomannans (Wheeler and Ratledge, 1988; Ortalo-Magne et al, 1995). The unusual cell wall gives rise to the distinctive staining property called acid-alcohol fastness. When stained by an aniline dye, such as carbol fuchsin, mycobacteria resist decolourisation with acid and alcohol because the dye forms a complex with the mycolic acid residues of the peptidoglycolipids in the cell wall and is held fast (Allen, 1992). This is the basis of the Ziehl-Neelsen (ZN) stain in which mycobacteria appear red whereas the background and non-acid-alcohol-fast organisms appear blue. Acid-alcohol fastness is characteristic of mycobacteria and the ZN stain is used to identify bacilli in the sputum and tuberculous lesions of infected patients.
In general, mycobacteria can be separated into two major groups: slow growers and fast growers. *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium bovis* and *Mycobacterium kansasii* are all slow growers, having generation times of fifteen to twenty hours (Smith, 1981). Two to three weeks of incubation at 37°C are required before visible colonies are formed on solid media. The colonies are generally off-white in colour, have a rough texture and are irregularly shaped. *Mycobacterium smegmatis*, *Mycobacterium phlei*, *Mycobacterium chelonae* and *Mycobacterium parafortuitum* are examples of fast growers. They have doubling times of around two hours (Smith, 1981) and visible colonies are formed after approximately forty-eight hours of incubation at 37°C.

At present, *Mycobacterium leprae* cannot be cultured in vitro. For experimental purposes, it is maintained in the armadillo. *Mycobacterium leprae* has an extremely slow doubling time, approximately eleven days in the mouse foot-pad. (Levy, 1976).

1.3. Pathogenicity of Mycobacteria.

*Mycobacterium tuberculosis* and *M. leprae* are the two pathogenic members of the genus *Mycobacterium*. They are the etiological agents of tuberculosis and leprosy respectively. *Mycobacterium bovis* and *Mycobacterium africanum* are capable of causing tuberculosis and are common pathogens in some areas of the world.

Tuberculosis occurs worldwide. It is estimated that fifty to sixty million people suffer from TB, three million die per year and that ten million new cases arise annually (Murray et al., 1989a). Tuberculosis causes approximately 6% of all deaths and at least 25% of all preventable deaths, making it the largest single infectious cause of mortality worldwide (Murray et al., 1989a). *Mycobacterium tuberculosis* infects one third of the population of the world (Sudre et al., 1992) and as such is considered the most common infectious agent of mankind.

Tuberculosis is typically a lung disease although *M. tuberculosis* can cause lesions in any tissue or organ of the body (Humphries et al., 1994). Humans are very susceptible to *M. tuberculosis* infection. The initial infection generates cell-mediated immunity and is usually
asymptomatic. The majority of the bacilli are killed but some viable organisms persist in the host and create a lifelong potential for disease. Normal, healthy individuals are resistant to tuberculosis disease and only 5-10% of those infected with tubercle bacilli will go on to develop active disease (Smith, 1981).

Leprosy afflicts ten to fifteen million people worldwide. One million new cases arise annually (Htoon et al, 1989) with the vast majority of cases being found in South-East Asia. There is a spectrum of disease and immunological responsiveness associated with leprosy. Cell-mediated immunity is well developed in the tuberculoid form of the disease. There is a progressive loss of cell-mediated immunity as the spectrum is traversed with lepromatous leprosy being characterised by an absence of cell-mediated immunity. There are few lesions containing few bacilli in tuberculoid leprosy but in lepromatous leprosy there are many lesions packed with bacilli (Ridley, 1974; Bullock, 1978). Leprosy is a debilitating disease, primarily of the skin and nerves. Nerve damage results in anaesthesia and disabilities but leprosy rarely causes death directly. Generally, secondary infections are responsible for the death of leprosy patients.

Approximately fifty species of non-pathogenic mycobacteria also belong to the genus *Mycobacterium* (Wayne and Kubica, 1986). Examples include *Mycobacterium smegmatis*, *Mycobacterium marinum*, *Mycobacterium gordonae*, *Mycobacterium xenopi* and *Mycobacterium malmoense*. The non-pathogenic mycobacteria are widely distributed in the environment, particularly in soil, dust and water, and exposure is unavoidable. Up to 50% of fecal samples from healthy individuals yield one or more environmental mycobacteria (Portaels et al, 1988). For those people who are immunosuppressed, the environmental mycobacteria can act as opportunist pathogens. The most common opportunists are *Mycobacterium kansasii*, *Mycobacterium avium*, *Mycobacterium intracellulare*, *Mycobacterium scrofulaceum* and *Mycobacterium ulcerans*. *Mycobacterium avium*, *M. intracellulare* and *M. scrofulaceum* are together known as the MAIS complex because they are phenotypically and genetically similar. The expanding population of immunocompromised individuals, that has come with the increase in transplants and HIV infection, has transformed these mycobacteria from environmental opportunists into frequent pathogens. When the opportunist pathogens are inhaled, a pulmonary disease develops that is clinically and pathologically identical to that produced by *M. tuberculosis*.
Mycobacteria that are ingested cause disease in the gastrointestinal tract which is typified by diffuse jejunal thickening and enlarged nodes without necrosis (Barnes et al, 1993).

1.4. Pathology of Tuberculosis.

Understanding of the pathology of tuberculosis has mainly come from work done with animals, particularly rabbits and guinea pigs (Lurie, 1964; Dannenberg, 1989 and 1991; Smith and Weigeshaus, 1989). There are two patterns to tuberculosis disease, depending on whether the infection is acquired for the first time (primary TB) or if it is acquired in a person who has been previously infected (secondary TB).

*Mycobacterium tuberculosis* most frequently enters the body through the lungs. Tubercle bacilli are contained in droplets that have been dispersed into the air by sneezing, coughing and speaking of infectious TB patients. Only droplets less than 5μm in diameter, containing 1-3 bacilli, reach the alveoli. Larger particles are eliminated by the mechanical barriers of the upper airways and the mucociliary system (Lurie, 1964). Alveolar macrophages rapidly ingest the bacilli. Whether *M. tuberculosis* can now establish an infection in the lung depends on both the virulence and viability of the bacilli and the inherent microbicidal ability of the alveolar macrophage that has ingested them (Dannenberg, 1991). If the bacteria survive the initial defences of the macrophage, unimpeded intracellular multiplication takes place. However, for tubercle bacilli, even the maximum rate of multiplication is slow and so the increase in the numbers of bacteria is also slow. Thus, the appearance of symptoms may take several weeks.

Multiplication of the tubercle bacilli is thought to lead to the death of the alveolar macrophage with the subsequent release of the bacteria (Rook, 1990). The presence of large numbers of bacilli causes an inflammatory response and monocytes enter the site, probably attracted by chemotactic factors from the released bacteria and the complement component C5a. These new, immature cells readily ingest the bacilli but cannot inhibit or destroy them as they have not yet been activated. The bacilli therefore continue to multiply (Dannenberg, 1991). *Mycobacterium tuberculosis* disseminates from the initial site of infection via the lymphatics and
enters the bloodstream. Tubercle bacilli are subsequently seeded to all organs of the body (Sheffield, 1994).

A cellular immune reaction develops after four to eight weeks, probably when the antigenic load reaches a critical level (Smith and Welgeshausen, 1989; Dunlap and Briles, 1993). Once cell-mediated immunity (CMI) develops, collections of activated T-cells and macrophages form granulomas that wall off *M. tuberculosis*. Granulomas are a characteristic of primary tuberculosis disease. Microorganisms tend to be localised in the centre of the granuloma, activated macrophages surround the pathogen and T-cells and monocytes surround the macrophages. Cytokines are released which activate the surrounding cells and recruit additional cells. The centre of the granuloma is nutrient-deficient and has a low oxygen tension which, along with digestion by activated macrophages, results in the death of the majority of bacilli (Dannenberg, 1991). Small numbers of viable pathogens do remain within the granuloma but for the majority of individuals (approximately 90%) the infection with *M. tuberculosis* is arrested at this point (Sheffield, 1994). These individuals are not infectious and cannot spread the disease to other people. However, they do face a lifelong risk of developing tuberculosis again from the few remaining live bacilli. The primary infection with *M. tuberculosis* is often asymptomatic. However, individuals will show delayed-type hypersensitivity (DTH) reactions to proteins found in the tubercle bacillus and this can be an indication of past *M. tuberculosis* infection.

In a small proportion of people (5-10% of individuals with a primary infection) cellular immunity fails to control and kill the bacilli. The disease progresses and there is extensive tissue damage, mainly due to DTH reactions. DTH is an adverse immunological reaction to components of the tubercle bacillus, that is mediated by T-cells and cytokines (Dannenberg, 1991). Tumour necrosis factor, interleukin-1 (Rook, 1988) and cytotoxic T-cells (Kaufmann, 1988b) have been reported to be responsible for the necrosis which is a consequence of the DTH reaction. DTH is beneficial to the host with poor CMI because it kills bacilli-laden, non-activated macrophages and nearby tissues, thereby eliminating the intracellular environment which is so favourable for bacterial growth. However, many bacilli still survive and are ingested by other non-activated macrophages where they again multiply (Dannenberg, 1991). DTH reactions are thus perpetuated and, if untreated, the host will eventually die from the effects of excessive tissue destruction and
consequent secondary infections.

Reactivation of the disease (secondary TB) occurs when host resistance is impaired. This may be due to immunosuppression from any cause, including illness, old age or malnutrition. Reactivation occurs in about 10% of infected persons. Of those, 5% develop secondary TB within the first two years after infection (Dunlap and Briles, 1993). Alternatively, secondary pulmonary TB can be the result of a new infection from an exogenous source which is also thought to trigger reactivation of dormant bacilli (Dunlap and Briles, 1993).

Secondary TB is characterised by the occurrence of extensive necrosis due to the strong DTH reaction generated by the interaction of mycobacterial antigen and sensitised T-cells. The cells surrounding the focus of infection die and the caseous material becomes surrounded by macrophages and T-cells to form a granuloma. Liquefaction of the caseous necrotic centre of the granuloma is one of the most harmful immune responses of secondary tuberculosis. It is a by-product of DTH and involves the hydrolysis of protein, lipid and nucleic acid components of caseous tissue by the hydrolytic enzymes of macrophages (Dannenberg, 1991). Liquefaction perpetuates the disease as it is an excellent environment for growth of the tubercle bacilli. Macrophages do not survive in liquefied caseous material and so are completely ineffective in controlling the extracellular multiplication of mycobacteria within a cavity. The bacilli multiply extensively and the large antigenic load becomes increasingly toxic to the surrounding tissues because of DTH reactions. The walls of nearby bronchi are thought to become necrotic and they rupture to form a cavity. The tubercle bacilli and the debris of liquefied and caseous tissue are discharged into the airway reaching other parts of the lung and the external environment. Such patients are said to have open tuberculosis and are highly infectious. At present, there is no therapeutic agent to prevent liquefaction but the appropriate antituberculosis drugs can reduce the number of viable bacilli and, in time, arrest the disease (Dannenberg, 1991).

1.5. Immunity to *M. tuberculosis*.

The great majority of individuals who are exposed to *M. tuberculosis* do not develop disease. This indicates that the host has effective mechanisms of protection. Resistance of the host...
to infection and disease comprises both natural (innate) and acquired immunity. When tubercle bacilli first enter the lungs they are rapidly phagocytosed by resident alveolar macrophages. It is at this point that natural immunity within the macrophage can be instigated to determine the fate of the bacteria. This process takes place well before an antigen-specific acquired immunity can develop.

a. Natural Immunity.

It has long been noted that individuals vary in their susceptibility to tuberculosis. For example, there is a 31.4% chance of an identical twin contracting TB when the other twin has the disease. For siblings and non-identical twins that chance is only 14.9% (Comstock, 1978). It has also been observed that Jews of Eastern European descent are unusually resistant to TB, that black Americans are twice as likely to develop TB as compared to white Americans and that Eskimos have a twenty-fold higher risk of contracting TB than white Americans (Stead et al, 1993; Bates and Stead, 1993). Indeed, Crowle and Elkins (1990) found that macrophages from black American donors tended to be more permissive for tubercle bacilli in vitro than those from white American donors. Thus, there are strong indications of genetic factors determining the host's susceptibility to tuberculosis disease.

Studies in mice have indicated that the early phase of natural resistance to mycobacteria is controlled by a single, dominant, autosomal gene on chromosome 1, which is termed \( Bcg \) (Denis et al, 1986; Forget et al, 1991; Schurr et al, 1991). Evidence was collected for a similar gene existing in humans (Schurr et al, 1991) and it was mapped to the telomeric end of chromosome 2q. The \( Bcg \) gene was found to markedly reduce the intracellular growth of mycobacteria within macrophages (Stach et al, 1984; Goto et al, 1989; Denis et al, 1990; Brown et al, 1995). This genetic resistance was expressed by mature tissue macrophages independently of T- and B-cells and Natural Killer cells (Buschman et al, 1989; Skamene, 1994). There is a candidate gene for \( Bc g \) which has been termed \( Nramp \), for Natural resistance associated macrophage protein (Vidal et al, 1993; Vidal et al, 1995). \( Nramp \) has been isolated from both mice and humans (Vidal et al, 1993; Barton et al, 1994; Cellier et al, 1994). Recently, full length clones of \( Nramp \) were sequenced and this provided evidence that the function of \( Nramp \) was related to signal transduction.
for macrophage priming and activation (Barton et al, 1994).

The Bcg gene is present in the mouse in two allelic forms: susceptible (Bcg°) and resistant (Bcg'). It was found to be tightly linked or identical with two other host-resistance genes, Ily and Lsh, which determine murine resistance to Salmonella typhimurium (Plant and Glynn, 1979) and Leishmania donovani (Bradley et al, 1979) respectively. Undoubtedly other genes exist that confer disease resistance on the host but they have yet to be characterised.

b. Acquired Immunity.

Acquired resistance results from specific immune responses by cells of the immune system. Acquired resistance can be divided into two categories: antibody-mediated (humoral) immunity and cell-mediated immunity (CMI).

On entering the body, tubercle bacilli are rapidly phagocytosed by macrophages. Once the pathogen is in an intracellular environment, antibody-mediated immune responses cannot play a part in the resistance to infection. However, it has been reported that there is an immunoglobulin M response followed by an increased titre of immunoglobulin G after tuberculosis infection (Grange, 1984; Sanchez et al, 1994). Raised immunoglobulin A levels (Skvor, 1979) and high levels of immunoglobulin E (Anders et al, 1989) have also been observed in patients with pulmonary TB. The role of these antibodies in resistance to tuberculosis infection is not clear, but it is possible that they may play a part in the opsonisation of tubercle bacilli thus promoting phagocytosis by mononuclear phagocytes. It has also been shown that coating of virulent tubercle bacilli with specific antibody enhances phagosome-lysosome fusion in non-activated murine macrophages (Armstrong and D'Arcy Hart, 1975).

It is believed that the effective immunological response to M. tuberculosis is almost entirely cell-mediated (Mackaness, 1971b). In fact, individuals with defective cellular immunity are much more susceptible to tuberculosis disease and are more likely to have a disseminated form of TB such as miliary TB or extrapulmonary TB (Dunlap and Briles, 1993).

One of the effector cells in CMI is the macrophage. It is dependent on interaction with activated T-cells for efficient bacterial killing. The processing of tubercle antigens for presentation to T-cells is an important function of the macrophage. If the antigen is processed in
the endosomal compartment of the macrophage, it associates with MHC class II molecules (Dunlap and Briles, 1993). Alternatively, mycobacterial antigens that gain access to the cytoplasm of the macrophage associate with MHC class I molecules (Kaufmann, 1991; McDonough et al, 1993; Pfaider et al, 1993). The MHC-antigen complex is expressed on the surface of the macrophage and, depending on the class of the MHC molecule, two different specific T-cell subsets are stimulated. Although exceptions exist, there seems to be a predilection of the two T-cell subsets for different functions. CD4+ T-cells (T4+, T8- in humans and L3T4+, Lyt2- in mice) recognize MHC class II molecules and preferentially secrete cytokines. CD8+ T-cells (T4-, T8+ in humans and L3T4-, Lyt2+ in mice) recognize MHC class I molecules. They preferentially lyse the cell that is expressing tubercle antigen complexed to the MHC class I molecule. Thus, the expression of different MHC-antigen complexes by the macrophage determines the T-cell response.

Production of cytokines by CD4+ T-cells is of obvious benefit to the infected host. It ensures activation of macrophages that can kill *M. tuberculosis*, the maturation of monocytes into macrophages and the recruitment of additional monocytes. It has been demonstrated that, in mice, CD4+ T-cells can be subdivided according to their cytokine pattern. TH1 cells preferentially produce interleukin-2, interleukin-12 and interferon-γ. TH2 cells primarily secrete interleukin-4, interleukin-5, interleukin-6 and interleukin-10 (Mosmann et al, 1986; Hatzigeorgiou et al, 1993). There is now strong evidence that a similar segregation exists in humans too (Parronchi et al, 1991; Romagnani, 1991; Cocks et al, 1993). The TH1 type cytokines cause inflammatory responses and DTH reactions (Cher and Mosmann, 1987) whilst the TH2 type cytokines are thought to be important in stimulating B-cells to produce certain types of antibody. For example, interleukin-4 induces B-cells to make immunoglobulin G1 and immunoglobulin E (Stevens et al, 1988).

For some infections it has been shown that the resistance of mice can be related to the subset of CD4+ T-cells that is induced (Scott et al, 1988; Heinzel et al, 1989). It has been observed that TH1 cells are associated with protective immunity in murine *M. leprae* infection (Walker et al, 1992) and in virulent *M. tuberculosis* infection (Orme et al, 1993b). In humans, the CD4+ T-cells induced by mycobacterial infection secreted a high level of interferon-γ but
little or no interleukin-4 (Haanen et al, 1991; Mutis et al, 1993). They are thus of the TH1 type. This would allow an inflammatory response rather than an inappropriate antibody response to develop to the mycobacterial infection.

It is thought that cytokines made by the tubercle antigen-presenting macrophage control the differentiation of uncommitted helper T-cell precursors into either type of effector cell. Interferon-γ (Chensue et al, 1995) and interleukin-12 (Mannetti et al, 1993) tend to induce T-cells of the TH1 type whereas interleukin-4 and interleukin-10 tend to convert T-cells into the TH2 type (Swain et al, 1990). Macrophages have been shown to produce interleukin-12 on infection with M. tuberculosis (D’Andrea et al, 1992; Cooper et al, 1995) which would allow a beneficial TH1 type response to develop. However, mycobacterial cell wall components have also been reported to stimulate macrophages to produce interleukin-10 (Barnes et al, 1992a) which would inhibit TH1 cell activity.

CD8+ T-cells with specificity for mycobacterial antigens have been identified in both the murine and human immune system. These T-cells have been shown to produce low levels of interferon-γ (de Libero et al, 1988). CD8+ T-cells have also been found to be capable of lysing autologous macrophages presenting mycobacterial antigens (Chiplunkar et al, 1986; de Libero et al, 1988; Kaufmann, 1988a). In addition, mycobacterium-specific CD4+ T-cells have been found to exist which possess cytotoxic activity for macrophages infected with mycobacteria (Mustafa and Godal, 1987; Pithie et al, 1992; Mutis et al, 1993). Stimulation of cytotoxic T-cells might benefit the host by allowing the destruction of those macrophages that are incapable of killing M. tuberculosis, thereby releasing the bacteria into the extracellular environment for uptake by more efficient macrophages (Kaufmann, 1988a; Kaufmann, 1989). Alternatively, the process of macrophage death might be linked to the killing of M. tuberculosis. Apoptosis of heavily infected human monocytes has been shown to result in a three-fold loss of viability of intracellular M. bovis BCG (Molloy et al, 1994). Apoptosis, mediated principally by CD4+ T-cells, is in fact believed to account for the bulk of the extensive cell death that occurs in a granuloma (Orme et al, 1993a).

Both the CD4+ and CD8+ T-cell subsets are likely to be important in the protective immune response to M. tuberculosis. It has been reported that there is a significant increase in
the CD4+/CD8+ T-cell ratio in patients with TB compared to normal subjects (Kuo and Yu, 1993). Mice that were depleted of either CD4+ T-cells or CD8+ T-cells and were then infected with M. bovis BCG or M. tuberculosis showed increased numbers of bacteria compared to normal animals, which indicates an inability to control the infection (Muller et al., 1987; Kaufmann, 1989; Kaufmann et al., 1989; Ladel et al., 1995). More severe effects were observed in CD4+ T-cell-deficient mice and abrogation of both T-cell subsets did not worsen the disease as compared with CD4+ depletion alone. In the mouse, CD4+ T-cells therefore have a greater role to play in immunity to mycobacterial infection. This is believed to be the case in humans too. Failure of the CD4+ T-cell response, as seen in people infected with HIV, contributes to progressive primary infection and to reactivation of endogenous mycobacteria (Slutkin et al., 1988).

The majority (more than 90%) of T-cells possess antigen receptors comprised of a heterodimer of α and β subunits. It is these cells that bear the major burden of protecting the host against mycobacterial disease (Orme et al., 1993a). However, there is a small proportion of T-cells that recognise antigen via a γδ receptor. The role of γδ T-cells is not well understood. The majority of the γδ T-cells lack the CD4 and CD8 molecules that participate in MHC recognition but they do respond to mycobacterial antigens (O’Brien et al., 1989; Born et al., 1990). It has also been found that immunisation of mice with M. tuberculosis stimulates a significant proportion of mycobacteria-reactive γδ T-cells (Janis et al., 1989). When peripheral blood leukocytes from normal individuals were stimulated *in vitro* with mycobacterial lysates, a marked expansion of γδ T-cells was seen to occur. An increase from less than 10% of total T-cells to more than 40% was observed (Kaufmann, 1991). Healthy contacts of tuberculosis patients were shown to have an increased number of γδ T-cells compared to healthy non-contacts and persons with TB (Ueta et al., 1994). This would indicate that γδ T-cells are involved in immunity to M. tuberculosis. γδ T-cells can produce certain cytokines (interleukin-2, interleukin-4 and interferon-γ) and can express cytolytic activities (Janis et al., 1989; Barnes et al., 1992b; Kaufmann and Young, 1992). For example, when γδ T-cells were stimulated with M. tuberculosis they produced interleukin-2 and showed specific killing of macrophages infected with M. tuberculosis (Munk et al., 1990). However, mice that were depleted of γδ T-cells did not show any adverse effects when infected with M. bovis BCG whereas α/β T-cell-depleted mice had an exacerbated infection.
These results suggest that, in the presence of \( \alpha/\beta \) T-cells, \( \gamma/\delta \) T-cells are not essential for antimycobacterial immunity.

Neutrophils are among the first cells to arrive at the site of infection with *M. tuberculosis* (Bloch, 1948). In mice, an intraperitoneal injection of *M. bovis* BCG was shown to induce a significant influx of neutrophils (Appelberg, 1992). Human macrophages secrete interleukin-8, an attractant for neutrophils, on phagocytosis of *M. tuberculosis* (Friedland et al, 1992). In return, neutrophils can release a chemotactic factor in response to mycobacteria that attracts macrophages (Anthony et al, 1983). It has been reported that the neutrophils of patients with active TB have increased adherence properties (Bass et al, 1981) and an increased phagocytic capacity (Reiger et al, 1979). This indicates that neutrophils become activated in tuberculosis. Neutrophils are capable of killing *M. tuberculosis* in vitro (Jones et al, 1970; Brown et al, 1987) and may therefore have a role in defence against mycobacteria.

Murine macrophages have been found to ingest neutrophils during an infection with mycobacteria (Silva et al, 1989). The in vitro antimycobacterial activity of murine peritoneal macrophages increased significantly when they had internalised granulocytic material (Silva et al, 1989). It was speculated that the ingestion of neutrophils provided the macrophages with molecules that enhanced their antimycobacterial activity (Silva et al, 1989). Thus, neutrophils may also participate indirectly in the control of mycobacterial infections.

Evidence has been presented that Natural Killer (NK) cells form an important part of the host response to mycobacteria. Interleukin-12 (Natural Killer cell stimulatory factor) was found to be produced by human macrophages infected with *M. tuberculosis* (D'Andrea et al, 1992; Cooper et al, 1995). Interleukin-12 can promote the synthesis of interferon-\( \gamma \) from NK cells (Tripp et al, 1993) and enhance the cytolytic activity of NK cells (Bancroft, 1993). It has been observed that NK cells activated with interleukin-2 (Bermudez and Young, 1991) and interleukin-12 (Bermudez et al, 1995) could induce mycobactericidal activities in human macrophages infected with *M. avium*. Activation of the macrophages was partly mediated by the NK cells secreting tumour necrosis factor (Bermudez and Young, 1991; Bermudez et al, 1995) and granulocyte-macrophage colony stimulating factor (Bermudez et al, 1995) but not interferon-\( \gamma \) (Bermudez et al, 1995). In addition, NK cells are capable of lysing host cells infected with...
mycobacterial pathogens which indicates functional similarities with specific cytolytic T-cells (Ab et al, 1990a; Ab et al, 1990b; Blanchard et al, 1992).

Thus, an overall picture of immunity to *M. tuberculosis* infection can be built up. At the site of mycobacterial multiplication, granulomas are formed which consist of neutrophils, mononuclear phagocytes at different maturation and activation states and T-cells. CD4+ T-cells preponderate, although some CD8+ T-cells are also present (Modlin et al, 1988; Cooper et al, 1989) with some γδ T-cells (Kaufmann, 1993). Under the influence of cytokines, the macrophages in the lesions are constantly activated to limit mycobacterial growth and new, highly effective monocytes are constantly attracted into the lesion. Protection from *M. tuberculosis* infection therefore depends on highly regulated interactions between T-cells and mononuclear phagocytes as well as other leukocytes, such as NK cells and neutrophils, within granulomatous lesions. It is a fine balance and if anything is disturbed bacterial multiplication and tissue destruction ensues, resulting in disease.

1.6. Resurgence of Tuberculosis.

Between the 1940s and the early 1980s the incidence of tuberculosis disease showed a significant decline in developed countries, mainly due to improved living conditions and effective chemotherapy (Dutt and Stead, 1993). Unfortunately, in the 1990s, this decline has markedly slowed or has even been reversed. In 1991, the number of TB cases reported in the United States rose by 18% as compared to those reported in 1985 (Barnes et al, 1993a). Between 1980 and 1991, the number of TB cases in New York City had risen by 143% (Barnes et al, 1993a). The increase has been predominantly among children and young adults, ethnic minorities and immigrants. These individuals are the most susceptible to tuberculosis disease; the children and young adults from primary infection and the ethnic minorities and immigrants from reactivation.

In developing countries, TB has never significantly declined. The huge incidence of tuberculosis disease in developing countries is mainly due to a greater than five-fold increase in population since 1850, with consequent malnutrition and poor living conditions (Kochi, 1994). In addition, there is a lack of chemotherapy and often non-effective BCG vaccination (Styblo,
Since the 1980s, however, tuberculosis has been on the increase (Elliot et al., 1990). For example, tuberculosis case rates doubled in Central Africa between 1990 and 1994 (Waliis and Eiller, 1994). It has also been estimated that, in 1992, 95% of all deaths from TB occurred in the developing world (Kochi, 1994).

Although there are a number of factors to be considered in the resurgence of TB, the greatest by far is the advent of the Acquired Immune Deficiency Syndrome (AIDS) which is caused by the Human Immunodeficiency Virus (HIV) (Pitchenik, 1990). *Mycobacterium tuberculosis* is the most common infection in HIV-infected patients worldwide. It was estimated that, in mid-1993, more than five million people were dually infected with HIV and *M. tuberculosis*; of these more than 75% lived in Africa (Kochi, 1994). Barnes *et al.* (1993a) report that 37% of HIV-infected, close contacts of TB patients develop disease within five months and that the risk of reactivation of TB in HIV-infected patients is 8-10% per year. Tuberculosis disease is associated with a declining CD4 count (Barnes *et al.*, 1993a). HIV specifically infects CD4+ T-cells and macrophages. This results in progressive depletion and dysfunction of CD4+ T-cells and abnormal macrophage function because of direct infection and a lack of macrophage activating factors from the CD4+ T-cells. As CD4+ T-cells and macrophages play a central role in immune defences against *M. tuberculosis* (section 1.5.), HIV infection facilitates the development of progressive, primary pulmonary TB, dissemination of *M. tuberculosis* to extrapulmonary sites and increased likelihood of reactivation of prior tuberculosis infection (Barnes *et al.*, 1993a). HIV infection predominantly affects the fifteen- to forty-nine-year-old age group and its impact has been greatest in developing countries where the prevalence of TB is also high in this age group.

The HIV epidemic has provided a highly susceptible population for the rapid spread of TB and has also increased disease transmission to persons with or without HIV infection. In developed countries, the recent increase in homelessness and poverty, particularly in the cities (Bloom and Murray, 1992), and the increase in immigration from countries where TB is common has also contributed to the rise in disease incidence and transmission.

A compounding factor in the resurgence of tuberculosis is the development of drug resistant *M. tuberculosis*, which makes treatment and cure more difficult. The Centers for Disease Control in Atlanta, U.S.A., conducted a nationwide survey of drug resistance among all TB
cases reported during the first three months of 1991. Overall it was found that 14.9% of patients had organisms resistant to at least one antituberculosis drug and 3.3% had organisms resistant to both isoniazid and rifampicin (Pozsik, 1993). Antituberculosis therapy is long term (lasting at least six months) and failure to complete therapy can lead to the selection of drug resistant bacteria. In 1990, reports received by the Centers for Disease Control from TB control programmes showed that approximately 24% of patients failed to complete therapy within a twelve month period and in some areas the figure was as high as 55% (Pozsik, 1993).

In the middle of this century, the decline in the incidence of tuberculosis in developed countries led to a widespread perception that TB was no longer an important public health problem. However, this view has now changed and there is a very urgent need for new antituberculosis drugs and a more effective vaccine to prevent a devastating tuberculosis epidemic. Central to this is a better understanding of the disease process and the host's immune response to M. tuberculosis.

1.7. The Activated Macrophage.

Macrophages are a heterogeneous population of cells which belong to the mononuclear-phagocyte system. The cells in this system all originate from a common precursor stem cell in the bone marrow and have similar morphological and functional properties. The mononuclear-phagocyte system includes circulating monocytes and tissue macrophages (Lowrie, 1983). Monocytes in the circulation are not destined for any particular tissue when they leave the bone marrow and their migration to different tissues appears to be a random phenomenon in the absence of localised inflammation (van Furth et al, 1979). They settle in numerous tissues where they undergo final differentiation into resident macrophages, possessing different characteristic features in each tissue. An estimate of the lifespan of macrophages in human tissues has been obtained from studies in patients receiving a bone marrow transplant and is considered to be approximately three months (Thomas et al, 1976).

The first cell to come into contact with tubercle bacilli in pulmonary infection is the alveolar macrophage. This cell is crucial in the development of resistance to tuberculosis
infection because it is responsible for killing the bacteria and activating T-cell function (section 1.5.). When macrophages are infected with mycobacteria they synthesise pro-inflammatory cytokines including tumour necrosis factor-α, interleukin-1α and -1β, interleukin-6, interleukin-8 and granulocyte-macrophage colony stimulating factor (Blanchard et al., 1991; Newman et al., 1991; Friedland et al., 1992; Michelini-Norris et al., 1992). Whole mycobacteria are not needed for the stimulation of cytokine production by macrophages. The lipoarabinomannan of M. tuberculosis has been shown to induce interleukin-1, interleukin-6 and tumour necrosis factor-α synthesis by macrophages (Moreno et al., 1989; Barnes et al., 1992a). Thus, on mycobacterial infection macrophages produce cytokines to ensure T-cell expansion and further macrophage activation, maturation and recruitment.

For optimal inhibition and killing of tubercle bacilli, resident macrophages must become activated. Mackaness (1971a) used the term "activation" to describe increased activity against ingested pathogens. Activated macrophages can be recognised functionally (Rhodes and Bennedsen, 1979) and morphologically (van der Rhoe et al., 1979). Activated macrophages are larger, display pronounced ruffling of the plasma membrane, have an increased capacity for adherence and spreading on surfaces, have increased pseudopods, have a higher metabolic rate and have an increased content of pinocytic vesicles, lysosomes and enzymes compared to resting macrophages (Cohn, 1978; Sharma and Remington, 1981; Adams and Hamilton, 1984; Edwards and Kirkpatrick, 1986; Pierangeli and Sonnenfeld, 1989). In addition, activated macrophages are more efficient at performing different functions such as phagocytosis, digestion, enzyme secretion and antigen processing (Dannenberg and Tomashefski, 1988). The biochemical changes that are observed following activation of the macrophage involve increased cell protein content and adenosine triphosphate content, increased glucose oxidation and oxygen consumption, increased hydrogen peroxide release and hexose monophosphate shunt activity (Karnovsky and Lazdins, 1978).

Activated macrophages display an increased surface expression of MHC class II molecules (van Furth, 1990). They also have an increased number of cell surface receptors for the Fc component of antibody and the C3b fraction of complement (Tsuda et al., 1976). This would significantly enhance phagocytosis of opsonised microorganisms into activated cells. However,
BCG-activated murine macrophages have been shown to have reduced expression of Fc receptors and mannose-specific receptors (Ezekowitz et al, 1981). In addition, activation with interferon-\(\gamma\) can down-regulate mannose/fucose receptors on human macrophages (Mokoena and Gordon, 1985) and suppress binding by the complement receptors, CR1 and CR3, of C3b- and C3bi-coated erythrocytes (Wright et al, 1986). In non-activated macrophages it has been demonstrated that mannose receptors (Bermudez et al, 1991; Schlesinger, 1993) and the complement receptors CR1, CR3 and CR4 (Schlesinger et al, 1990; Bermudez et al, 1991; Hirsch et al, 1994) are important in mediating phagocytosis of mycobacteria. It might be expected, therefore, that activated macrophages would be less efficient at phagocytosing mycobacteria. Indeed, Stokes et al (1993) found that BCG-activated murine macrophages were less able to bind \(M.\) \textit{tuberculosis} than non-activated murine macrophages. Interferon-\(\gamma\)-activated human macrophages have a decreased ability to phagocytose \(M.\) \textit{tuberculosis} (Douvas et al, 1985) and \(M.\) \textit{leprae} (Schlesinger and Horwitz, 1991). Thus, binding of mycobacteria to activated macrophages is far from optimal.

The activation of macrophages can be viewed as a sequential process. Two stages are involved: priming and triggering. All the morphological, metabolic and secretory changes can occur after a non-specific inflammatory stimulus, for example the phagocytosis of a pathogen (priming). The macrophage acquires microbicidal activity only after specific cytokine-mediated stimulation, which occurs following the exposure to the pathogen (triggering) (Cohn, 1978).

Macrophage activation during \textit{in vivo} infection is restricted to the site of infection (Dannenberg, 1989). It is most prominent within granulomas induced by the T-cell-mediated immune response. Activated macrophages in the centre of granulomas display the highest degree of bacterial killing and histochemically they appear to be the most abundant in lysosomal enzymes (Dannenberg, 1989).

1.8. Activation of Macrophages for \textit{in vitro} Antimycobacterial Activity.

Most humans infected with \(M.\) \textit{tuberculosis} do not develop disease (section 1.4.) which suggests that macrophages can kill tubercle bacilli \textit{in vivo}. It is generally accepted that resistance to tuberculosis is mediated by specific T-cells and is expressed by activated macrophages (Crowle
and May, 1981; Crowle, 1988). It should therefore be possible to activate macrophages in vitro by using the right combination and concentration of cytokines. However, activating macrophages for in vitro antimycobacterial activity and defining which cytokines are responsible has proven to be difficult.

Efforts to activate macrophages for tuberculocidal activity in vitro have involved incubating non-activated macrophages with crude T-cell supernatants or with purified recombinant cytokines. Some success has been achieved with murine cells. Walker and Lowrie (1981) activated mouse peritoneal macrophages in vitro to kill M. microti, the natural agent of tuberculosis in voles, by using supernatants from PPD-stimulated lymphocytes. Subsequently, Khor et al (1986) observed that recombinant interferon-γ was sufficient to activate mouse peritoneal macrophages in vitro to kill M. microti. Chan et al (1992) found that recombinant interferon-γ, when in combination with recombinant tumour necrosis factor-α, was able to activate mouse peritoneal macrophages to kill M. tuberculosis Erdman.

Generally, experiments with murine cells have shown stasis and not killing of tubercle bacilli. Mouse peritoneal macrophages (Sypek et al, 1993) and bone marrow-derived macrophages (Flesch and Kaufmann, 1987) were activated by recombinant interferon-γ to inhibit the growth of M. bovis BCG. Interferon-γ was also able to activate peritoneal macrophages (Rook et al, 1986a) and bone marrow-derived macrophages (Flesch and Kaufmann, 1987) to inhibit M. tuberculosis H37Rv. However, M. tuberculosis Middelburg was resistant to inhibition by bone marrow macrophages activated with interferon-γ (Flesch and Kaufmann, 1987), suggesting that there is heterogeneity in the susceptibility of tubercle strains to the antimycobacterial activity of macrophages.

Numerous other cytokines have been tested in murine macrophages. Tumour necrosis factor-α (Kaufmann and Flesch, 1988; Rook, 1990; Sypek et al, 1993), interleukin-2, interleukin-4 (Kaufmann and Flesch, 1988; Rook, 1990), interleukin-6, colony stimulating factors and interferon-α and -β (Kaufmann and Flesch, 1988; Flesch and Kaufmann, 1990a and 1990b) have all been found not to stimulate antimycobacterial activity when added before infection.
Activation of macrophages can require more than one cytokine signal. The cytokines act in synergy to promote antimycobacterial activity. Interferon-γ and tumour necrosis factor-α have been shown to activate murine peritoneal macrophages to kill *M. tuberculosis* Erdman (Chan et al, 1992). These two cytokines also acted synergistically to stimulate tuberculostasis in bone marrow-derived macrophages (Flesch and Kaufmann, 1990a).

The timing of the addition of the cytokine to murine macrophages may influence its effect. Khor et al (1986) observed that adding interferon-γ prior to infection caused macrophages to kill *M. microti*. If the interferon-γ was added after the infection period the macrophages were bacteriostatic. Interferon-γ resulted in inhibition of the growth of *M. bovis* BCG inside murine macrophages when added prior to infection but it was less effective (Flesch and Kaufmann, 1990b) or ineffective (Sypek et al, 1993) when added after infection. In contrast, when interleukin-4 or interleukin-6 were added to murine bone marrow macrophages, after they had been infected with *M. bovis* BCG, these cytokines induced antimycobacterial functions (Kaufmann and Flesch, 1988; Flesch and Kaufmann, 1990b).

*Mycobacterium bovis* BCG-sensitised lymphocytes are capable of inducing antimycobacterial activity in cultured murine macrophages infected with BCG (Sypek et al, 1993). This effect was not due to the secretion of cytokines by the T-cells and required direct contact between the lymphocytes and the macrophages. It was postulated that the tumour necrosis factor-α associated with the T-cell membrane may have been responsible for the activation of macrophage antimycobacterial activity (Sypek et al, 1993).

The progress that has been made in activating murine macrophages for antimycobacterial activity has not been mirrored with human macrophages. Unfortunately, understanding murine macrophage tuberculocidal activity has shed little light on human macrophage tuberculocidal activity. The first report of human macrophages inhibiting the replication of *M. tuberculosis* came in 1981 (Crowle and May, 1981). It was found that supernatants collected from lymphocytes of immune donors, that had been stimulated with mycobacterial antigen *in vitro*, could activate homologous monocyte-derived macrophages to inhibit the replication of *M. bovis* BCG and *M. tuberculosis* Erdman. The identity of the cytokines in the supernatants of the stimulated lymphocytes was not established.
Recombinant cytokines, particularly interferon-γ, have since been tested for activation of human macrophage tuberculoicidal activity. In contrast to the mouse, interferon-γ appears unable to reproducibly activate human macrophages for antimycobacterial activity. It was found that human alveolar macrophages (Steele et al., 1986) and human monocytes (Rook et al., 1986a) were unable to consistently limit the growth of M. tuberculosis when activated with interferon-γ. However, interferon-γ treatment was demonstrated by Carvalho de Sousa and Rastogi (1992) to endow both monocytes and macrophages with significant tuberculostatic activity. Others have observed that interferon-γ given to monocyte-derived macrophages before (Robertson and Andrew, 1991) or at the time of (Denis et al., 1990) infection had no effect on the growth of M. tuberculosis. Interferon-γ treatment of human macrophages was even reported to enhance the replication of intracellular M. tuberculosis (Douvas et al., 1985; Meylan et al., 1992).

Of the other cytokines that have been tested, interleukin-1, interleukin-2, interleukin-3, interleukin-4, interleukin-6 and interferon-α have all been found not to alter the growth of M. tuberculosis inside human macrophages (Denis et al., 1990; Rook, 1990; Denis, 1991). Tumour necrosis factor-α was reported by Rook (1990) and Meylan et al. (1992) to have no effect on intracellular M. tuberculosis but Denis et al. (1990) observed that tumour necrosis factor-α slightly inhibited the growth of tubercle bacilli inside human macrophages.

Increased levels of the active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (calcitriol), have been found in the pleural fluids of patients with tuberculous pleuritis (Barnes et al., 1989). Calcitriol promotes the maturation and activation of human monocytes and macrophages (Amento et al., 1984; Mangelsdorf et al., 1984). When human macrophages are exposed to interferon-γ they develop a 1-hydroxylase which converts the circulating inactive form of calcitriol into the active 1-hydroxylated derivative. Rook et al. (1986b) cultured human monocytes for three days with the physiological concentration of calcitriol (10⁻⁷M) and found that these cells were capable of consistently inhibiting the intracellular growth of M. tuberculosis. Other calcitriol derivatives were not active at this concentration. When calcitriol and interferon-γ were included together in the cultures, the effects seen were additive and of similar magnitude resulting in greater than 60% inhibition of growth (Rook et al., 1986b). Crowle et al. (1987) also reported that calcitriol enabled human macrophages to slow or stop
intracellular *M. tuberculosis* replication, but only at a concentration of 4μg ml⁻¹ (approximately 10⁻⁵M). This is considerably higher than normal circulating concentrations (Schwartzman and Frank, 1987) but Crowie et al. (1987) postulated that this concentration could be reached in infectious granulomas as the activated macrophages can synthesise calcitriol from the precursor, which circulates at levels one thousand-times higher than those of active calcitriol.

Retinoic acid, a metabolite of vitamin A, causes monocytic differentiation (Hemmi and Breitman, 1987). Crowle and Ross (1989) reported that physiological concentrations of retinoic acid (10⁻⁷M), added before infection, lengthened the intracellular generation time of *M. tuberculosis* inside human macrophages and occasionally caused bacteriostasis. Thus, immunologically active vitamins may be involved in the activation of tuberculooidal mechanisms in human macrophages.

It is unlikely that, in vivo, one single chemical activates human macrophages for antimycobacterial activity. Indeed, all those tested in vitro have had little effect on their own. A combination of chemicals, at the correct concentration and in the correct sequence, might be more suitable for activating the full tuberculooidal mechanisms of human macrophages in vitro. To that effect, Denis (1991b) reported that incubating human monocytes for twenty-four hours with a combination of interferon-γ, tumour necrosis factor-α and calcitriol would activate them to kill *M. tuberculosis* over a seven day period.

1.8.1. Reasons for the Variation Shown Between Experiments.

The mouse does not constitute the best animal model for human tuberculosis. Susceptibility to the disease differs between mice and humans (Brown, 1983). Mice are more resistant to tuberculosis and the disease is characterised by the long-term persistence of a static population of bacteria (Rees and D'Arcy Hart, 1961). Indeed, Lowrie (1990) has suggested that the generation of mycobacteriostasis in activated murine macrophages in vitro may be the appropriate immune response for the mouse. This is in contrast to humans, where the primary infection leads to the existence of macrophages that possess tuberculooidal activity (section 1.4.).

In addition to physiological differences, there are also biochemical differences between
human and murine macrophages. For example, murine macrophages, unlike human macrophages, do not possess a 1-hydroxylase for the conversion of inactive calcitriol to the active form. Also, murine macrophages use products from the metabolism of arginine in tuberculocidal activity (section 1.9.). Human macrophages have not yet been conclusively shown to utilise these products. Thus, results obtained from murine macrophages cannot be used to explain the antmycobacterial activity of human macrophages, without serious reservations.

There is a great deal of variation experienced both within and between experiments with human macrophages. There is variation between macrophages obtained from a single individual and there is, of course, variation between macrophages obtained from different individuals. Donor-to-donor variation, which may have been due to inherent differences or environmental differences, was experienced in the experiments of Crowie and May (1981) and Rook et al (1986a). It has been demonstrated that different races of people have inherent differences in the ability of their macrophages to inhibit tubercle bacilli (Crowie and Elkins, 1990).

The source of the macrophages can affect the growth of tubercle bacilli. Obviously, there are morphological, metabolic and functional differences between circulating monocytes and tissue macrophages (Hocking and Golde, 1979; Rich et al, 1989). Hirsch et al (1994) found that human alveolar macrophages inhibited the replication of M. tuberculosis H37Ra to a greater extent than blood monocytes. Variability is also introduced by the use of differing periods of monocyte preculture before the addition of mycobacteria. It was observed that M. tuberculosis Erdman grew readily in freshly isolated blood monocytes and in monocyte-derived macrophages obtained after seven days in culture (Douvas et al, 1986). However, monocytes cultured for only three days prior to infection were less permissive for the mycobacteria (Douvas et al, 1988). The antmycobacterial activity of macrophages may be affected by the conditions of culture. Variation in serum is likely to be particularly problematic (Flesch and Kaufmann, 1987; Crowle and Elkins, 1990) but the use of serum-free culture could overcome this problem (Flesch and Kaufmann, 1987).

Clearly this amount of variation between experiments confuses the issue of whether human macrophages can be activated in vitro to inhibit or kill M. tuberculosis. Agreement should be reached as to the conditions of culture, the type of cell and the strain of mycobacterium that is
to be used in these experiments, so that worthwhile conclusions can be drawn.

1.8.2. Vaccination as a Means of Activating Macrophages.

In general, exposing non-activated macrophages to cytokines \textit{in vitro} fails to generate activated macrophages capable of killing \textit{M. tuberculosis} (section 1.8.). An alternative to this approach is to vaccinate subjects so that their macrophages become activated \textit{in vivo}. This technique has been used especially in animals, with varying results.

In an early study, peritoneal monocytes were taken from normal or BCG-vaccinated rabbits. The monocytes were then incubated with either \textit{M. tuberculosis}, \textit{M. avium} or \textit{M. phlei} but neither stasis nor killing of the mycobacteria was observed (Hanks and Evans, 1940). In contrast, other studies reported that monocytes from BCG-vaccinated guinea pigs, rabbits and mice inhibited the growth of intracellular tubercle bacilli (Suter, 1953; Berthrong and Hamilton, 1959; Maxwell and Marcus, 1968). However, all these experiments included streptomycin in the culture media, which may have had an antimicrobial effect on the intracellular mycobacteria.

More recently, Jackett et al (1981a and 1981b) demonstrated that guinea pigs vaccinated and boosted with \textit{M. bovis} BCG could kill a challenge dose of \textit{M. tuberculosis} \textit{in vivo}. This experiment was the basis for the development of a vaccination schedule that generated macrophages capable of killing \textit{M. tuberculosis} \textit{in vitro} (O'Brien and Andrew, 1991; O'Brien et al, 1991). Guinea pigs were vaccinated, boosted and challenged with \textit{M. bovis} BCG. When the alveolar macrophages were collected they were found to kill up to 40% of the original inoculum of both virulent and avirulent strains of \textit{M. tuberculosis} over twenty-four hours. Other vaccination schedules with \textit{M. bovis} BCG did not produce suitably activated macrophages.

Thus, the vaccination schedule developed by O'Brien and colleagues allowed the \textit{in vitro} study of antimycobacterial mechanisms in activated guinea pig macrophages. An added advantage was that the guinea pig is a better animal model for human TB. The guinea pig is just as susceptible to tuberculosis disease as humans and the pathology of the disease is similar to that found in man (Brown, 1983; Smith and Wiegeshaus, 1989).
1.9. Macrophage Antimycobacterial Mechanisms.

Inhibition or killing of tubercle bacilli by human macrophages has so far proved difficult to demonstrate in vitro (section 1.8.). Accordingly, mechanisms of macrophage antimycobacterial action in man are still poorly understood. In contrast, progress has been made in elucidating the mycobactericidal properties of murine macrophages because it is possible to activate these macrophages to kill mycobacteria in vitro (section 1.8.).

It is known that macrophages possess many antimicrobial mechanisms. These antimicrobial activities can be divided into those dependent on oxidative metabolism and those which are oxygen-independent. Both are discussed in the following sections.


Oxygen-dependent antimicrobial mechanisms are those which involve toxic forms of oxygen such as superoxide anions, hydrogen peroxide, singlet oxygen and the hydroxyl radical. These oxygen species accumulate during the respiratory burst that accompanies phagocytosis.

There is evidence for the involvement of peroxide in tuberculocidal activity (Andrew et al, 1985; Lowrie et al, 1985). For example, increased chemiluminescence was observed in monocytes from TB patients (Kitahara et al, 1979) which indicates an increased production of hydrogen peroxide and superoxide. Mitchison et al (1983) demonstrated that there was a correlation between virulence of tubercle strains in the guinea pig and in vitro resistance to peroxide. Jackett et al (1981a) found that peroxide-sensitive mutants of M. tuberculosis survived less well in guinea pigs than the peroxide-resistant parent strains. Finally, Walker and Lowrie (1981) observed that the addition of catalase abolished the in vitro killing of M. microti by activated murine macrophages.

However, when the role of peroxide was further investigated, particularly with in vitro models, it was concluded that macrophages did not require any products of the respiratory burst to kill or inhibit M. tuberculosis. Guinea pig macrophages were found to kill peroxide-resistant strains of M. tuberculosis just as readily as peroxide-susceptible strains (Lowrie et al, 1985; O'Brien and Andrew, 1991). It was demonstrated that peroxide-susceptible strains and peroxide-
resistant strains were equally effective in triggering a respiratory burst (O'Brien and Andrew, 1991; O'Brien et al, 1991), thus ruling out the possibility that peroxide-susceptible strains escaped maximum killing by not stimulating a respiratory burst. In addition, it has been demonstrated that scavengers of toxic oxygen products have no effect on the intracellular killing of tubercle bacilli in activated macrophages (Flesch and Kaufmann, 1988; O'Brien and Andrew, 1991; Chan et al, 1992).

Other evidence against a role for toxic oxygen products has come from cells that cannot mount a respiratory burst. Chan et al (1992) reported that a respiratory-deficient clone of the murine macrophage cell line, J774, was as capable of killing M. tuberculosis as the respiratory-sufficient parental clone.

Thus, the majority of the in vitro evidence indicates that respiratory burst products are not essential for macrophage tubercucoidal activity. There is additional, indirect evidence against a role for the respiratory burst in tubercucoidal activity. The phagocytosis of mycobacteria by complement receptors (section 1.7.) does not trigger the respiratory burst (Schlesinger et al, 1990). Mycobacteria can also interfere with the generation of toxic oxygen radicals. Low-molecular weight fractions of mycobacteria, including lipoarabinomannan (Sibley et al, 1988; Chan et al, 1991), sulpholipids (Pabst et al, 1988; Zhang et al, 1988) and phenolic glycolipid-1 (Chan et al, 1989), have been shown to be capable of inhibiting the respiratory burst.

It is still possible that oxygen-dependent mechanisms have a part to play in the overall antimycobacterial activity of macrophages. Indeed, it is highly probable that macrophages kill M. tuberculosis with a variety of toxic mechanisms and not one single, exclusive mechanism. However, it is not easy to explain the contradictory evidence for and against the involvement of respiratory burst products in macrophage tubercucoidal activity. The correlation between virulence and peroxide resistance (Mitchison et al, 1963) might be explained by assuming that resistance to peroxide is a manifestation of resistance to another macrophage tubercucoidal mechanism. Walker and Lowrie (1981) observed that catalase reversed the antimycobacterial activity of murine macrophages whereas Chan et al (1992) reported that catalase had no effect on tubercucoidal activity in murine macrophages. These opposite findings could be due to the different activation procedures used by the two groups, which may have induced different

1.9.2. Oxygen-independent Antimicrobial Mechanisms.

At present, it is thought that oxygen-independent antimicrobial mechanisms are more important in the killing of tubercle bacilli inside macrophages. Macrophages possess numerous oxygen-independent antimicrobial mechanisms and it is possible that several of them have a role to play in tuberculocidal activity.

a. Iron Sequestration.

Deprivation of iron results in slow growth, poor multiplication, stasis and even death of microorganisms (Wheeler and Ratledge, 1988). The availability of iron as a microbial nutrient can, therefore, play a critical role in infection (Kochan, 1973; Weinberg, 1978). Indeed, a normal host response to infection is to increase the production of transferrin, an unsaturated iron-binding protein. Transferrin can render serum bacteriostatic by limiting extracellular iron. Another iron-binding protein, lactoferrin, is found in mucosal secretions and has been shown to be bacteriostatic and bacteriocidal against a variety of microorganisms in vitro (Oram and Reiter, 1968; Arnold et al., 1980; Bortner et al., 1986).

It can be imagined that a decrease in the iron concentration of serum would have little effect on intracellular microbes, such as M. tuberculosis. However, serum transferrin was found to be bacteriostatic to tubercle bacilli (Kochan, 1973), being effective against both intracellular and extracellular mycobacteria (Kochan and Smith, 1965; Douvas et al., 1993). The inhibition of the growth of intracellular M. tuberculosis, due to iron limitation in serum, was found to be much greater in macrophages from vaccinated guinea pigs than in macrophages from normal guinea pigs (Kochan and Smith, 1965; Kochan, 1973). It has been reported that interferon-γ-activated
human monocytes down-regulate the number of transferrin receptors on their surface (Byrd and Horwitz, 1989) and down-regulate the concentration of intracellular ferritin, the major iron storage protein in these cells (Byrd and Horwitz, 1990). This decrease in intracellular iron availability was found to be responsible for the inhibition of *Legionella pneumophila* replication inside macrophages (Byrd and Horwitz, 1989 and 1990).

In addition to down-regulating transferrin receptors and ferritin concentrations, macrophages may use neutrophil-derived lactoferrin to decrease levels of intracellular iron. It has been observed that macrophages ingest neutrophils during chronic mycobacterial infections in the mouse (Silva *et al*, 1989). However, it is not clear whether limiting iron availability is an important defence mechanism against *M. tuberculosis*. Tubercle bacilli can produce mycobactins which transfer iron across lipid envelopes and exochelins which scavenge iron from the aqueous environment (Macham *et al*, 1975). The production of both mycobactin and exochelin was found to increase when strains of *M. tuberculosis* were incubated in media that contained transferrin or lactoferrin (Raghu *et al*, 1993). Whether iron sequestration has an effect on the *in vivo* survival of *M. tuberculosis* is therefore questionable.

b. Proteins and Peptides.

A large number of antimicrobial cationic proteins have been identified in the lysosomes of polymorphonuclear leukocytes (Odeberg and Olsson, 1975; Modrazakowski and Spitznagel, 1979; Elsbach and Weiss, 1983; Shafer *et al*, 1984; Ganz *et al*, 1985). These proteins are active at an alkaline or near neutral pH and their activity is mainly due to their cationic structure and not their enzyme activity (Odeberg and Olsson, 1975; Modrazakowski and Spitznagel, 1979; Elsbach and Weiss, 1983; Ganz *et al*, 1985).

During an infection, macrophages may obtain the cationic proteins by ingestion of neutrophils (Silva *et al*, 1989). Alternatively, macrophages may synthesise them. Antibacterial activity, due to cationic proteins, has been reported in macrophages (Patterson-Delafield *et al*, 1980; Lehrer *et al*, 1983). Two cationic proteins, isolated from rabbit alveolar macrophages, were shown to be active against a range of Gram-positive and Gram-negative bacteria (Patterson-Delafield *et al*, 1980; Lehrer *et al*, 1983). The optimum pH for antibacterial action
in vitro was between seven and eight, which is consistent with a role for the proteins immediately after phagocytosis. Rabbit alveolar macrophages have also been shown to contain a cationic protein known as cathepsin G (Andrew et al., 1983).

The role of cationic proteins in macrophage defence against *M. tuberculosis* has not been fully investigated. The two proteins isolated from rabbit alveolar macrophages (Patterson-Delafield et al., 1980; Lehrer et al., 1983) were not tested for tuberculocidal activity. However, certain cationic proteins from polymorphonuclear leukocytes, known as defensins, were shown to be active against *M. avium* (Ogata et al., 1992) and *M. fortuitum* (Lehrer et al., 1993). Proteins were found in homogenates of guinea pig peritoneal exudate monocytes that inhibited growth of tubercle bacilli (Kotani et al., 1962; Ramseier and Suter, 1964). Tuberculostatic activity was greater in monocytes from vaccinated animals. Three cationic proteins have also been isolated from the lysosomes of murine peritoneal macrophages (Hiemestra et al., 1993). These proteins were present in interferon-γ-activated cells and non-activated cells and they had a broad spectrum of antibacterial activity, which included activity against *M. fortuitum*. Evidence was found for additional microbialidal proteins and the synthesis of one of these proteins may have been induced by interferon-γ. Thus, cationic proteins are a potential tuberculocidal mechanism of macrophages, although these proteins have yet to be found in human monocytes (Ogata et al., 1992).

c. Degradation of Tryptophan.

Tryptophan is an amino acid essential for protein synthesis. The effectiveness of the degradation of tryptophan as an antimicrobial mechanism relies on the starvation of microorganisms of this amino acid. However, in most cases it results only in cytostasis (Byrne et al., 1986; Pfefferkorn et al., 1986). The degradation of tryptophan has been reported to inhibit the replication of *Toxoplasma gondii*, *Chlamydia psittaci* and *Leishmania donovani* in human monocytes and macrophages (Byrne and Carlin, 1989; Murray et al., 1989b).

Tryptophan degradation has been shown to be induced in interferon-γ-activated human alveolar macrophages (Murray et al., 1989b). Interferon-γ stimulates increased synthesis of the enzyme indoleamine 2,3-dioxygenase (IDO) which degrades tryptophan to n-formyl kynurenine.
and kynurenine (Carlin et al, 1987; Ozaki et al, 1987). However, tryptophan degradation was demonstrated not to have a role in the antimycobacterial activity of activated guinea pig alveolar macrophages. Addition of excess tryptophan did not reverse macrophage killing of *M. tuberculosis* and IDO activity was not increased in activated macrophages (personal communication from B.J. Roberts, Department of Microbiology and Immunology, University of Leicester; O'Brien, 1992).

d. Phagosomes and Lysosomes.

There are two features of phagosomes and lysosomes that are important in their antimicrobial activity: the environment within phagosomes is acidic and lysosomes contain antimicrobial enzymes that kill and degrade microorganisms. These two properties have been demonstrated to be effective against a wide range of microorganisms (Andrew et al, 1985) but their toxicity for mycobacteria has not been established.

1. Acid.

Microorganisms that are ingested by macrophages are contained within phagosomes. After phagocytosis, ATPase-dependent proton pumps accumulate in the phagosomal membrane (Pitt et al, 1992) which cause a decrease in pH. The pH of phagosomes is approximately 4.5 (Sprick, 1956; de Duve et al, 1978) and this would be expected to be unfavourable for bacterial growth. The susceptibility of mycobacteria to pH depends on the nature of the acid, the bacilli being more susceptible to organic acids (Dubos, 1950). However, mycobacteria seem to be able to inhibit the acidification of macrophage phagosomes. Viable *M. tuberculosis* and *M. avium* have been observed inside non-acidic phagosomes in non-activated human macrophages (Crowle et al, 1991). Only dead mycobacteria were found in acidic phagosomes. The pH of phagosomes formed around *M. avium* in non-activated murine bone marrow-derived macrophages was between 6.3 and 6.5 (Sturgill-Koszycki et al, 1994). It was reported that the phagosomal membranes surrounding *M. avium* and *M. tuberculosis* did not acquire subunits (Sturgill-Koszycki et al, 1994) or accessory proteins (Xu et al, 1994) of the proton pump which prevented acidification of the phagosome. Maintenance of the phagosomal pH around neutral would favour mycobacterial growth, as demonstrated by the ability of tubercle bacilli to multiply in the phagosomes of non-activated macrophages.
murine macrophages (Armstrong and D'Arcy Hart, 1971). Whether mycobacteria can inhibit the process of acidification in activated macrophages has not been investigated.

2. Lysosomal Enzymes.

Phagosomes fuse with lysosomes, in a process called phagosome-lysosome fusion, to allow digestion of the phagocytosed microorganism. Macrophages possess populations of lysosomes (Canonico et al, 1978; Lowrie et al, 1979; Andrew et al, 1985) which may contain different sets of enzymes. The fusion of these distinct populations may be under separate physiological controls (Lowrie et al, 1979). For example, the lysosomal subpopulations from rabbit alveolar macrophages had different tendencies to fuse with phagosomes containing M. bovis BCG (Andrew et al, 1985). Fusion of the different lysosomal populations would also be predicted to have varying consequences for the ingested microbe, if each lysosomal population contains different enzymes (Lowrie et al, 1979).

Increased lysosomal enzyme activity has been found in macrophages from tuberculous lesions (Dannenberg, 1974), in monocytes of untreated pulmonary tuberculosis patients (Jaswai et al, 1993) and in M. bovis BCG-induced alveolar macrophages (Sorber et al, 1973; Lowrie et al, 1979). Katoh (1981) reported that the phagocytosis of tubercle bacilli by murine peritoneal macrophages increased the activities of acid phosphatase and β-glucuronidase. However, this increase in lysosomal enzyme activity may only be a consequence of macrophage activation (section 1.7.) rather than a tuberculocidal mechanism.

Macrophages possess numerous lysosomal enzymes which are capable of degrading proteins, lipids, polysaccharides, peptidoglycans and nucleic acids (Lowrie et al, 1979; Andrew et al, 1983; Darte and Beaufay, 1983). Lysozyme has been found to be bacteriostatic to mycobacteria in vitro but very large, probably physiologically unrealistic, concentrations were required for killing (Myrvik et al, 1953a and 1953b). Mycobacteria, including M. tuberculosis, were observed to be resistant to digestion by other lysosomal enzymes (Stahelin et al, 1956; Ginsburg, 1979). Mycobacterium tuberculosis cell wall components have also been shown to be capable of inhibiting certain lysosomal enzymes (Weiss and Singer, 1953; Lowrie and Andrew, 1988).
Thus, mycobacteria appear to be resistant to the action of the lysosomal enzymes *in vitro*. However, *M. tuberculosis* and *M. bovis* BCG have been reported to avoid exposure to the lysosomal enzymes by inhibiting phagosome-lysosome fusion in non-activated murine macrophages (Armstrong and D'Arcy Hart, 1971 and 1975) and in non-activated human macrophages (Clemens and Horwitz, 1995). This would imply that the lysosomal contents are toxic *in vivo*. The bacilli were able to multiply in the unfused phagosomes (Armstrong and D'Arcy Hart, 1971). Viable tubercle bacilli had the ability to inhibit phagosome-lysosome fusion but dead bacteria did not (Armstrong and D'Arcy Hart, 1971; Clemens and Horwitz, 1995). A relationship was demonstrated between the virulence of the mycobacteria and inhibition of phagosome-lysosome fusion in non-activated murine peritoneal macrophages. A virulent strain of *M. tuberculosis* (H37Rv) inhibited fusion but macrophages infected with the less virulent *M. tuberculosis* H37Ra and *M. bovis* BCG showed progressive phagosome-lysosome fusion (D'Arcy Hart and Armstrong, 1974). Inhibition of phagosome-lysosome fusion may thus be an important factor in mycobacterial virulence.

The mechanism(s) used by *M. tuberculosis* to inhibit phagosome-lysosome fusion has not been deduced. *Mycobacterium tuberculosis* is known to produce ammonia (Ratledge, 1982) which can inhibit phagosome-lysosome fusion (Gordon *et al.*, 1980). Phagosome-lysosome fusion is believed to occur upon acidification of the phagosome (Mellman *et al.*, 1986). It is therefore possible that phagosome-lysosome fusion is inhibited because ammonia, which is excreted by the ingested mycobacterium, counteracts the acidification of the phagosome (Mellman *et al.*, 1986). The exclusion of the proton pump from the phagosome membrane (Sturgill-Koszycki *et al.*, 1994; Xu *et al.*, 1994) would similarly inhibit phagosome-lysosome fusion in cells infected with mycobacteria.

Biosynthesis of inhibitory substances may be crucial to prevent phagosome-lysosome fusion because dead *M. tuberculosis* failed to inhibit fusion (Armstrong and D'Arcy Hart, 1971). Sulfatides, which are found on the surface of *M. tuberculosis*, have been shown to antagonise the fusion of lysosomes with phagosomes (Goren, 1982). Mycobacterial surface components may be involved in the inhibition of phagosome-lysosome fusion as fusion can be induced by opsonising *M. tuberculosis* and *M. microti* with antibodies, thus masking the bacterial surface (Armstrong and
When phagosome-lysosome fusion was induced by opsonisation, *M. tuberculosis* could still multiply in the phagolysosomes of non-activated macrophages (Armstrong and D'Arcy Hart, 1975). Thus, *M. tuberculosis* was resistant to attack from the lysosomal constituents of murine non-activated macrophages. It has been observed that macrophages can be activated to overcome the inhibition of phagosome-lysosome fusion by mycobacteria. Human macrophages were incubated for twenty-four hours with macrophage colony stimulating factor before being infected with *M. avium* (Duzgunes et al, 1993). Using this protocol phagosome-lysosome fusion was found to be induced. Interferon-γ-activated mouse peritoneal macrophages also demonstrated enhanced phagosome-lysosome fusion, compared to non-activated macrophages, when infected with *M. leprae* (Sibley et al, 1987). However, phagosome-lysosome fusion was still inhibited in human macrophages infected with *M. tuberculosis*, even when the cells had been pre-treated with interferon-γ (Clemens and Horwitz, 1995).

The lysosomal contents of activated macrophages have not been tested for antimycobacterial activity (Sibley et al, 1987; Duzgunes et al, 1993), although a correlation was observed between inhibition of lysosomal fusion and survival of *M. avium* in macrophages from *Bcg*⁺ and *Bcg*⁻ mice (section 1.5.) (de Chastellier et al, 1993). The percentage of phagosome-lysosome fusion was found to be twice as high in *Bcg*⁺ macrophages and the percentage of viable bacilli was observed to be twice as low in *Bcg*⁺ macrophages as compared to *Bcg*⁻ macrophages. Thus, mycobacteria may be susceptible to killing by the lysosomal enzymes of activated macrophages.

McDonough *et al* (1993) recently proposed an alternative mechanism by which mycobacteria could avoid the lysosomal contents of non-activated macrophages. They infected human and murine macrophages with *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra and *M. bovis* BCG and found that approximately 85% of the bacteria were residing in phagolysosomes two hours after infection. Four days after infection it was reported that the numbers of phagolysosomes containing *M. tuberculosis* H37Rv and H37Ra, but not *M. bovis* BCG, were reduced by half. Thus, *M. tuberculosis* H37Rv and H37Ra had the capacity to escape from phagolysosomes inside human and murine macrophages. Only *M. tuberculosis* H37Rv was able to
multiply once it had escaped from the phagolysosome. This suggests that the ability to escape the phagolysosome is a determinant of mycobacterial virulence.

It was claimed that these results (McDonough et al, 1993) did not contradict those of Armstrong and D'Arcy Hart (1971 and 1975) because the extent of phagosome-lysosome fusion was monitored at different times by the two groups. Thus, McDonough et al (1993) observed a high percentage of phagosome-lysosome fusion two hours post-infection but four days later the number of phagolysosomes containing *M. tuberculosis* had decreased by half. In contrast, Armstrong and D'Arcy Hart (1971 and 1975) recorded low levels of phagosome-lysosome fusion one or two days after macrophage infection. McDonough et al (1993) therefore proposed that Armstrong and D'Arcy Hart (1971 and 1975) had missed the phagosome-lysosome fusion event that occurs soon after the infection of macrophages with mycobacteria and had only observed mycobacteria that had escaped from the phagolysosome. However, Clemens and Horwitz (1995) have recently reported their failure to repeat the findings of McDonough et al (1993). They found that, in human macrophages, there was very little fusion of lysosomes with *M. tuberculosis*-containing phagosomes three hours and one day after infection. In addition, *M. tuberculosis* was not observed free in the cytoplasm at any time up to five days post-infection (Clemens and Horwitz, 1995).

Unfortunately, there is confusion at present as to where mycobacteria reside inside the macrophage and whether they avoid the lysosomal contents. It is clear that additional research is required to clarify the role of lysosomes in the tuberculocidal activity of macrophages.

e. Production of Reactive Nitrogen Intermediates.

Reactive nitrogen intermediates (RNI) are generated from the amino acid L-arginine by the enzyme nitric oxide synthase (NOS) (Marletta, 1993). NOS catalyses the oxidation of one of the two chemically equivalent guanidino nitrogens of L-arginine to nitric oxide (Hibbs et al, 1988; Marletta et al, 1988; Stuehr et al, 1989). Nitric oxide is a highly reactive free radical and can be rapidly oxidised to form other RNI such as peroxynitrite and nitrogen dioxide. Nitrite and nitrate are produced as the stable oxidative end products (Marletta et al, 1988). Macrophages possess an inducible NOS (iNOS) which is regulated at the transcriptional level (Lorsbach et al,
When expressed, iNOS is located in the cytoplasm. It is NADPH- but not calcium-dependent (Marietta et al., 1988) and requires tetrahydrobiopterin as a cofactor (Tayeh and Marietta, 1989; Sakai et al., 1992).

Tumour cells are susceptible to RNI produced by activated murine macrophages and this cytotoxicity can be reproduced by incubating the tumour cells with nitric oxide gas alone (Hibbs et al., 1988; Stuehr and Nathan, 1989). Nitric oxide binds to catalytically active iron-sulphur centres (Lancaster and Hibbs, 1990; Pollat et al., 1990) causing the release of iron ions and the formation of iron-nitrosyl complexes (Hibbs et al., 1984), thus inactivating the enzymes. The ribonucleotide reductase (Kwon et al., 1991; Lepoivre et al., 1991), aconitase (Drapier and Hibbs, 1986), NADH/succinate oxidoreductase and ubiquinone oxidoreductase (Granger et al., 1980; Granger and Lehninger, 1982; Stuehr and Nathan, 1989) of tumour cells are all inhibited by nitric oxide. The inactivation of these enzymes leads to inhibition of mitochondrial respiration and DNA synthesis which are characteristics of macrophage-mediated tumour cell cytotoxicity (Klostergaard et al., 1991).

RNI are also toxic to microorganisms and it is likely that nitric oxide acts in a similar fashion. Iron-sulphur centres in Clostridium botulinum have been shown to react with nitric oxide to form iron-nitrosyl complexes (Reddy et al., 1983) and nitric oxide production by murine macrophages has been detected by its reaction with the iron-sulphur protein, ferredoxin, of Clostridium pasteurianum (Stuehr et al., 1989). In addition, nitric oxide was capable of deaminating deoxynucleosides, deoxynucleotides and intact DNA at physiological pH in vitro (Wink et al., 1991). Salmonella typhimurium was treated with three nitric oxide-releasing compounds and similar DNA damage was observed (Wink et al., 1991).

The in vitro production of RNI by activated murine macrophages has been shown to be effective against numerous pathogens, causing stasis or even killing of the microbes. Cryptococcus neoformans (Granger et al., 1988; Cameron et al., 1990), Entamoeba histolytica (Lin and Chadee, 1992), Ehrlichia risticii (Park and Rikihisa, 1992), Francisella tularensis (Anthony et al., 1992; Green et al., 1993), Herpes simplex virus (Karupiah and Harris, 1995), Leishmania (Mauel et al., 1991; Assreuy et al., 1994; Cillari et al., 1994), Listeria monocytogenes (Beckerman et al., 1993; Bermudez, 1993), Schistosoma mansoni (James and Glaven, 1989),
Toxoplasma gondii (Adams et al, 1990), Trypanosomes (Vincendeau and Daulouede, 1991; Munoz-Fernandez et al, 1992a; Vincendeau et al, 1992) and Vaccinia virus (Melkova and Esteban, 1994; Harris et al, 1995; Karupiah and Harris, 1995) have all been shown to be susceptible to the RNI generated by cultured murine macrophages.

The synthesis of RNI has also been shown to be essential for antileishmanial activity in vivo. Mice that possessed a disruption in the iNOS gene were observed to be highly susceptible to Leishmania major infection. The animals subsequently developed severe visceral disease (Wei et al, 1995). In contrast, wild-type mice were highly resistant to the infection with L. major and the disease was found to heal spontaneously (Wei et al, 1995).

It is clear that RNI are toxic to a diverse range of microbes. Some of these are obligate or facultative intracellular organisms, others are extracellular. The intracellular microbes occupy a variety of subcellular locations, such as phagosomes or phagolysosomes, or they are free in the cytoplasm. The fact that nitric oxide is a small molecule that would be able to traverse membranes of the host cell and microorganism (Karupiah and Harris, 1995) is the likely explanation for its ability to affect both intracellular and extracellular pathogens.

The iNOS of murine macrophages can produce large amounts of RNI (nmoles min⁻¹ mg protein⁻¹) for a prolonged period (twenty-four to forty-eight hours) when induced by cytokines (Henry et al, 1993). Maximum induction is obtained with two cytokines. Combinations of interferon-γ, tumour necrosis factor-α (Ding et al, 1988; Drapier et al, 1988; Deng et al, 1993), interleukin-2 (Cox et al, 1992; Munoz-Fernandez et al, 1992a; Deng et al, 1993) and macrophage migration inhibition factor (Cunha et al, 1992) are capable of synergistically activating murine macrophages to synthesise RNI in vitro. Conversely, interleukin-4 (Liew et al, 1991; Oswald et al, 1992a; Melillo et al, 1994), interleukin-10 (Cunha et al, 1992; Oswald et al, 1992a), interleukin-13 (Doherty et al, 1993) and transforming growth factor-β (Ding et al, 1990; Oswald et al, 1992a) can inhibit the induction of RNI synthesis in activated cells.

Alternatively, LPS (Lorsbach and Russell, 1992; Cunha et al, 1993) or staphylococcal exotoxins (Fast et al, 1991) can synergise with cytokines to induce the expression of iNOS in murine macrophages. The phagocytosis of group B streptococci (Goodrum et al, 1994), Leishmania (Corradin and Mauel, 1991; Cunha et al, 1993), Listeria (Celada and Schreiber,
1987), Staphylococcus aureus (Cunha et al, 1993; Goodrum et al, 1994) and heat-killed M. bovis BCG (Stuehr and Marietta, 1987) was also found to stimulate a high level of RNI production by cytokine-activated murine macrophages. It has been demonstrated that the generation of nitric oxide upon ingestion of group B streptococci (Goodrum et al, 1995), Leishmania (Corradin et al, 1991), Schistosoma mansoni (Oswald et al, 1992b) and Toxoplasma gondii (Langermans et al, 1992) is dependent on the production of tumour necrosis factor-α by the infected macrophages. This cytokine acts as an autocrine second signal, thereby enhancing the expression of macrophage iNOS.

The relatively large amounts of nitric oxide that are generated to kill microorganisms also have deleterious effects on the macrophage and the surrounding tissues by inducing intracellular iron loss and inactivation of enzymes (Drapier and Hibbs, 1988; Vanin et al, 1993; Green and Nacy, 1994). It therefore makes sense that only those cells that have encountered a pathogen synthesise RNI and that cytokines can regulate the production.

Mycobacteria are also susceptible to RNI generated by activated murine macrophages. It has been demonstrated that cultured murine macrophages synthesise RNI when infected with M. bovis BCG (Flesch and Kaufmann, 1991; Barrera et al, 1994), M. leprae (Adams et al, 1991) and M. tuberculosis (Denis, 1991a; Chan et al, 1992). In addition, the generation of RNI has been shown to be important in resistance to tuberculosis infection in vivo (Chan et al, 1995). It was observed that mortality, bacterial burden and pathological tissue damage increased when mice infected with M. tuberculosis were fed inhibitors of iNOS (Chan et al, 1995).

Bcg^ macrophages (section 1.5.) have been found to produce three times as much nitrite as Bcg^ macrophages, due to an enhanced level of iNOS expression on stimulation with interferon-γ (Barrera et al, 1994). The Bcg^ macrophages had greater bacteriostatic activity against M. bovis BCG compared to Bcg^ macrophages and this activity was reversed when an inhibitor of iNOS was included in the medium. Originally, it was hypothesised that the candidate gene for Bcg, Nramp, (section 1.5.) might be involved in the delivery of nitrates to the phagolysosome of the infected macrophage, where the acid environment would mediate the conversion, via nitrites, to toxic nitric oxide (Vidal et al, 1993). However, it is now believed that Nramp functions as a signal transductant for macrophage priming and activation (Barton et al, 1994).
The susceptibility of *M. avium* to RNI appears to vary according to the strain. Doi and colleagues (1993) found that *M. avium* strain Mino was inhibited by nitric oxide generated from acidified nitrite. *Mycobacterium avium* strain 101 (Bermudez, 1993) and strain 26291 (Appelberg and Orme, 1993) were resistant to RNI synthesised by cytokine-activated mouse peritoneal macrophages. In fact, the inhibition of these strains by murine macrophages was demonstrated to be independent of RNI.

The existence of an inducible NOS in human macrophages has not yet been convincingly demonstrated and there is much conflicting data in the literature. It is possible to activate the iNOS of human hepatocytes for RNI production (Nussler et al, 1992; Geller et al, 1993; Meilouk et al, 1994). The cytokines required to induce RNI production in human hepatocytes correspond to the stimuli required for the generation of RNI in murine macrophages. However, it has been reported that these same cytokines were not able to induce iNOS or RNI production by human macrophages in vitro (Murray and Teitelbaum, 1992; Padgett and Pruett, 1992; Dumarey et al, 1994; Schneemann et al, 1994; Zembala et al, 1994).

When cultured human macrophages were infected with *Chlamydia psittaci*, *Leishmania donovani*, *Toxoplasma gondii* (Murray and Teitelbaum, 1992), *Cryptococcus neoformans* (Cameron et al, 1990) or *Ehrlichia chaffeensis* (Barnewall and Rikihisa, 1994), RNI were not generated nor were they required for the killing of these pathogens. Human macrophages have a significantly lower activity of 6-pyruvoyl-tetrahydrobiopterin synthase compared to other human cells and murine cells (Werner et al, 1990). It can therefore be postulated that the inability of human macrophages to produce RNI is due to a lack of the cofactor, tetrahydrobiopterin. The addition of tetrahydrobiopterin to cytokine-stimulated murine cells resulted in an increase in RNI production (Sakai et al, 1992; Sakai and Milstein, 1993). However, this treatment did not result in any detectable accumulation of RNI by stimulated human macrophages (Sakai and Milstein, 1993; Schneemann et al, 1993; Michaliszyn et al, 1995).

Elevated plasma and urine nitrate levels are found in septic patients (Hegesh and Shiloah, 1982; Ochoa et al, 1991) or cancer patients being treated with interleukin-2 (Hibbs et al, 1992; Ochoa et al, 1992), suggesting that human macrophages may possess a functional iNOS in vivo. Contrary to the majority of published observations, a limited number of researchers have
demonstrated that cultured human macrophages can produce RNI. Certain tumour cells were reported to induce RNI production by human macrophages in vitro (Martin and Edwards, 1993; Thomsen et al, 1993; Zembala et al, 1994), although other tumour cells were found not to elicit an RNI response (Harwix et al, 1992; Zembala et al, 1994). It is possible that human macrophages may not respond to all tumour cells in an identical manner.

Interferon-γ, tumour necrosis factor-α (Munoz-Fernandez et al, 1992b) or LPS (Hunt and Goldin, 1992; Leibovich et al, 1994) were shown to be capable of inducing RNI production in human macrophages. When human macrophages were infected with a virulent strain of *M. avium* (CIPT 14031096), RNI were generated (Dumarey et al, 1994). However, infection with *M. tuberculosis* H37Rv and *M. smegmatis* did not lead to RNI production (Dumarey et al, 1994). *Trypanosoma cruzi* (Munoz-Fernandez et al, 1992b) and an avirulent strain of *M. avium* (LR/149) (Denis, 1991c) were reportedly killed by RNI produced by cytokine-activated human macrophages. In contrast, Bermudez (1993) found that RNI were not produced by cytokine-activated human macrophages and were not necessary for the killing of *M. avium* strain 101.

The involvement of RNI in the antimycobacterial activity of murine macrophages is well documented. However, whether RNI have a role to play in human macrophage antimycobacterial activity has not been established. This confusing situation needs to be clarified with more research.

f. Ornithine Metabolism.

Ornithine is the first amino acid in an enzymatic pathway that leads to the formation of cytotoxic aminoaldehydes (Bachrach and Persky, 1964; Morgan, 1985; Levitz et al, 1990). The first part of the pathway is shown in Fig. 1.1. The polyamines, spermine and spermidine, are ubiquitous in eukaryotes and prokaryotes, being essential for the control of cell proliferation and differentiation (Heby, 1981; Morgan, 1985).

An enzyme called polyamine oxidase (PAO) can catalyse the deamination of spermine and spermidine to aminoaldehydes. There are two types of PAO, one is found in the sera of ruminants (Bovine Plasma PAO) and the other is found in a wide range of mammalian tissues (Tissue PAO) and in the sera of pregnant women (Retroplacental Serum PAO) (Morgan, 1980 and 1985). The
Fig. 1.1. The Formation of Polyamines from the Amino Acid, Ornithine.

Ornithine Decarboxylase

ORNITHINE → PUTRESCINE

CARBON DIOXIDE

DECARBOXYLATED
S-ADENOSYL
METHIONINE

SPERMIDINE

DECARBOXYLATED
S-ADENOSYL
METHIONINE

Spermidine Synthase

SPERMINE
Two forms of PAO produce different end-products from spermine and spermidine (Fig. 1.2.).

Bovine serum PAO acts on the primary amino groups of spermine to form an aminodialdehyde.

Tissue PAO cleaves spermine at the secondary amino groups to produce an aminoaldehyde (Morgan, 1981 and 1985).

N,N^1-Bis(3-propanal)-1,4-diaminobutane, N-(4-aminobutyl)-3-aminopropanal and 3-aminopropanal are all aminoaldehydes (Fig. 1.2.). These aminoaldehydes have been demonstrated to be toxic to bacteriophages, bacteria (including *M. tuberculosis*), fungi, protozoa, helminths, nematodes and eukaryotic cells (Bachrach et al, 1963; Bachrach and Persky, 1964; Morgan, 1985; Ferrante et al, 1986; Levitz et al, 1990). The aminoaldehydes were also shown to be capable of killing *Plasmodium falciparum* parasites inside red blood cells (Rzepczyk et al, 1984).

In the majority of experiments, Bovine Plasma PAO has been used to generate the toxic aminoaldehydes *in vitro*. The aminoaldehydes produced by Bovine Plasma PAO were found to bind to the amino groups of purine and pyrimidine bases in DNA (Eilon and Bachrach, 1969). They had an increased affinity for DNA with a high G-C content, presumably because a G-C pair has two free amino groups in contrast to the one amino group of an A-T pair. The mechanism of toxicity of the aminoaldehydes is therefore believed to be an inhibition of nucleic acid synthesis and consequently protein synthesis. Mycobacteria have G-C rich DNA and it is interesting to note that *M. tuberculosis* was nearly twice as sensitive to oxidised spermine than the other tested bacteria (Bachrach and Persky, 1964).

The generation of toxic aminoaldehydes is a potential macrophage antilmycobacterial mechanism. The conversion of ornithine to putrescine by ornithine decarboxylase (ODC) is the rate limiting step in the formation of the aminoaldehydes. It has been reported that when macrophages are activated there is a subsequent increase in ODC expression. For example, ODC activity can be induced in macrophage-like cell lines (RAW 264) and monocytic cell lines (U937) with LPS (Taffet and Haddox, 1985; Yukloka et al, 1989). LPS and interferon-γ induced ODC activity in murine peritoneal macrophages and human monocytes (Messina et al, 1990). Tumour necrosis factor was found to stimulate the accumulation of ODC by human monocytes and macrophages (Kaczmarek et al, 1992).
Fig. 1.2. The Production of Aminoaldehydes by the Two Forms of Polyamine Oxidase (PAO).

**Bovine Plasma PAO.**

\[
\begin{align*}
\text{Spermine + oxygen + water} & \rightarrow N,N_{1}\text{-Bis(3-propanal)-1,4-diaminobutane} \\
& \quad + \text{ammonia + hydrogen peroxide} \\
\text{Spermidine + oxygen + water} & \rightarrow N\text{-}(4\text{-aminobuty1)-3-aminopropanal} \\
& \quad + \text{ammonia + hydrogen peroxide}
\end{align*}
\]

**Tissue PAO/Retroplacental Serum PAO.**

\[
\begin{align*}
\text{Spermine + oxygen + water} & \rightarrow \text{Spermidine + 3-aminopropanal} \\
& \quad + \text{hydrogen peroxide} \\
\text{Spermidine + oxygen + water} & \rightarrow \text{Putrescine + 3-aminopropanal} \\
& \quad + \text{hydrogen peroxide}
\end{align*}
\]
The cell wall material of *M. bovis* BCG has been observed to increase ODC expression in a murine macrophage cell line (J774) and in murine peritoneal exudate macrophages (Nichols and Prosser, 1980). Thus, mycobacterial infection may upregulate the expression of ODC in macrophages. An increase in ODC would allow the macrophage to increase production of the aminoaldehydes for antimycobacterial activity. The levels of ODC also regulate another function of macrophages. Kierzenbaum *et al* (1987) found that murine macrophages lost the capacity to phagocytose Trypanosoma cruzi when ODC was inhibited. An increase in ODC may therefore enhance the phagocytosis of mycobacteria by macrophages.

Generation of aminoaldehydes for macrophage antimicrobial activity could also be regulated at the point where the polyamines are deaminated. Macrophages possess the Tissue-type PAO (Morgan, 1985). The aminoaldehyde products of Tissue PAO have been shown to be toxic to *Dirofilaria immitis* microfilariae, *Schistosoma mansoni* schistosomula (Ferrante *et al*, 1986), Trypanosoma musculi and a leukaemia cell line (Storer *et al*, 1988). In all these cases hydrogen peroxide was found not to be the toxic moiety. PAO levels were observed to be elevated in activated macrophages compared to non-activated macrophages (Morgan and Illei, 1980; Morgan *et al*, 1980). Morgan and colleagues (1980) immunised rabbits with *M. bovis* BCG and found that the alveolar macrophages had an increased content of PAO per cell. Thus, there is compelling evidence for the aminoaldehydes being involved in macrophage antimycobacterial activity.

### 1.10. Determining the Nature of the Antimycobacterial Activity of the Macrophage.

Understanding the mechanism(s) by which activated macrophages kill *M. tuberculosis* may facilitate progress in the areas of TB prevention and therapy. Most researchers opt to study the macrophage *in vitro* to try to elucidate antimycobacterial mechanisms (section 1.8. and 1.9.). An alternative approach is to study the properties of tubercle bacilli before and after passage through animals.

Passaging involves administering a mycobacterial inoculum to an animal and then recovering the bacilli after a certain length of time. The procedure selects for those bacteria that
are highly resistant to the antimycobacterial activity of macrophages in vivo (Crowle, 1988). By testing the susceptibility of the harvested mycobacteria to various toxic substances, the method by which macrophages kill *M. tuberculosis* in vivo may be inferred. For example, if after being passaged the population of bacteria are more resistant to the action of cationic proteins (section 1.9.) than the original bacterial population, then it can be concluded that cationic proteins are important in antimycobacterial activity in vivo.

There are two main advantages to using the technique of passaging. One is that a protocol for activating macrophages to kill *M. tuberculosis* in vitro is not required. The second is that the killing of the mycobacteria takes place in vivo. The results of passaging are therefore more relevant for clinical purposes. However, there are also disadvantages to passaging. It can never be used in humans. Thus, it can only be hypothesised that any discoveries made by passaging *M. tuberculosis* through animals apply to man. In addition, differences in susceptibility to toxic agents will only be detected if the recovered mycobacteria express the relevant virulence determinant in vitro. In other words, factors that affect the survival of the mycobacteria in vivo can only be detected in toxicity tests if the bacilli also possess them in vitro. Finally, testing the susceptibility of mycobacteria to toxic substances allows differences between the initial population and the passaged population to be determined. Thus, only those factors that have undergone some change within the population can be detected, identifying them as targets of macrophage tuberculocidal activity. There will of course be other factors that do not undergo any fundamental change whilst the bacteria are being passaged but which are potentially susceptible to macrophage products. For example, the synthesis of DNA and protein is thought to be inhibited by aminoaldehydes (section 1.9.). The technique of passaging does not therefore provide evidence for all the mechanisms that may be used by macrophages to kill *M. tuberculosis*.

1.11. *Mycobacterial Survival in Conditions of Acid Stress.*

There are many instances in which mycobacteria may find themselves in an acidic environment. Free-living mycobacteria (section 1.3.) will encounter naturally acidic conditions in soil and water (Collins *et al*, 1984). In fact, acidity in soil and water is becoming more
widespread because of the frequent occurrence of pollutants such as acid rain, sewage and fertilisers. The use of the antimycobacterial drug, pyrazinamide, which can only exert its toxic effects in acidic environments (Konno et al., 1967; Crowle et al., 1989; Chan, 1994), indicates that mycobacteria are exposed to acidic conditions during infection.

The tuberculous lesion possesses an anaerobic environment which results in the formation of carbonic acid in the aqueous phase. Dubos (1953) has also postulated that organic acids are excreted into the lesion by the anaerobic metabolism of the surrounding cells. A potentially acidic environment that the mycobacteria may encounter in vivo is the macrophage phagosome (section 1.8.). Phagosomal pH can be as low as 4.5 (Sprick, 1956; de Duve et al., 1978) but mycobacteria have been reported to reside in non-acidic phagosomes in non-activated macrophages (Crowle et al., 1991; Sturgill-Koszycki et al., 1994). It has been demonstrated that mycobacteria can inhibit the activity of the ATPase-dependent proton pump, thereby preventing acidification of the phagosome (Sturgill-Koszycki et al., 1994; Xu et al., 1994). The acidity of phagosomes in activated macrophages infected with mycobacteria has not been established.

The ability to survive low-pH environments, whether in a tuberculous lesion or the macrophage phagosome, may therefore be crucial for mycobacteria to establish an infection. Mycobacteria are capable of maintaining viability at acidic pH. This is demonstrated by the ability of M. bovis to survive the extreme acidity of the stomach and to cause infection when ingested in milk (Fanning, 1994).

Distinctions can, in fact, be made between the slow growing and fast growing mycobacteria (section 1.2.) in their ability to multiply on acidic media (Chapman and Bernard, 1962; Portaels and Pattyn, 1982). In general, the slow growers multiply optimally over a narrower pH range than the fast growers (Portaels and Pattyn, 1982). In addition, the optimal pH for the growth of slow growers is typically between 5.8 and 6.5 whereas for fast growers it is generally between 7.0 and 7.4 (Portaels and Pattyn, 1982). The slow growing mycobacteria, which include many pathogens, therefore prefer slightly acidic conditions for growth.

Mycobacterium tuberculosis has a very narrow range for optimal growth in vitro that is between pH 5.8 and 6.5. The bacteria are not capable of multiplying at a pH less than 5.4 (Portaels and Pattyn, 1982). However, M. smegmatis is capable of growth between pH 4.6 to 7.4.
Of all the fast growing mycobacteria that were tested, *M. smegmatis* had the widest pH range for optimal growth, from pH 5.0 to 7.4 (Portaels and Pattyn, 1982).

It has been demonstrated that there are variations between strains of *M. tuberculosis* in their ability to survive exposure to acidic pH (Jackett et al., 1978). The viability of strain H37Ra was least affected by a ninety minute incubation at pH 4.0. However, the viability of strains 79112, 12846 and 79499 decreased by an approximately equal amount (Jackett et al., 1978). Resistance to acidity does not therefore correlate with virulence among these strains.

In order to survive acidity, mycobacteria, in common with all other bacteria, must be able to maintain their internal pH within a range suitable for viability (Booth, 1985). A number of mechanisms may be employed, both constitutive and inducible, which will regulate cytoplasmic pH when the external pH decreases. The buffering capacity of the cytoplasm, which is due to the amino acid side-chains of proteins, and proton transport are protective mechanisms that are considered to be constitutively expressed by the bacterial cell (Booth, 1985). In addition, several enzymes have been found to be preferentially induced when the external pH is acidic (Gale and Epps, 1942; Gale, 1946; Stephenson, 1949). These include lysine, arginine and ornithine decarboxylases, which are able to counteract external acidity by producing basic compounds, and the degradative enzyme, formic hydrogenlyase, which destroys acidic material. However, these mechanisms normally fail to regulate cytoplasmic pH when the external pH falls below 4.0. Under these conditions, the plasma membrane becomes permeable, lesions occur in ribosomes and DNA and enzymes denature (Pryzbylski and Witter, 1979; Raja et al., 1991).

Recently, a superimposing genetic response has been discovered that can protect a bacterial cell against much stronger acidic conditions (external pH less than 4.0). The response is induced on exposure to a mildly acidic environment (pH 5.0 to 6.0). A number of bacteria have been shown to possess this adaptive response. They are *Escherichia coli* (Goodson and Rowbury, 1989), *Salmonella typhimurium* (Foster and Hall, 1990), *Listeria monocytogenes* (Kroll and Patchett, 1992), *Streptococcus mutans, Enterococcus hirae* (Belli and Marquis, 1991), *Aeromonas hydrophila* (Karem et al., 1994) and root nodule bacteria (O'Hara and Glenn, 1994).

An acid adaptive response is an obvious advantage to both free-living bacteria and pathogenic ones as it allows survival in conditions that would normally be lethal. For example, S.
typhimurium can survive within macrophage phagolysosomes (Carrol et al, 1979; Fields et al, 1986) where the pH is approximately 4.5 (Sprick, 1956; de Duve et al, 1978) and yet pH values around 4.0 are bactericidal to unadapted cells in vitro (Foster and Hall, 1990). The acid adaptation response may therefore have an important role in the pathogenesis of S. typhimurium.

The discovery of an acid adaptive response in mycobacteria would lead to a greater understanding of the ability of M. tuberculosis to survive within the host and cause disease. It was therefore decided to test in this thesis whether mycobacteria possess an adaptive response to acid stress. For this purpose, the experiments that described the adaptation response of S. typhimurium (Foster and Hall, 1990) were repeated with M. smegmatis. The main reason for choosing to repeat these experiments was that the acid adaptation response of S. typhimurium is the best characterised (Foster, 1993; Lee et al, 1994). A description of the acidification tolerance response (ATR) of S. typhimurium follows.

Growth of S. typhimurium occurs between a wide range of pH values (pH5.0 to 9.0) (Foster, 1993). As a facultative intracellular pathogen, S. typhimurium can encounter environments that are below pH5.0, examples of which include the stomach, polluted water and the macrophage phagolysosome (Foster, 1993). In the absence of protective mechanisms, cell death occurs when the intracellular pH falls below 5.4 (Foster and Hall, 1991). However, the ATR can effectively protect S. typhimurium from the lethal effects of acid conditions by preventing a major decrease in internal pH (Foster and Hall, 1990).

The ATR in S. typhimurium involves two stages: a pre-acid shock stage which is induced at pH5.8 and a post-acid shock stage which is induced at or below pH4.5 (Foster, 1991). The pre-shock stage induces a pH homeostasis system which acts to maintain the intracellular pH as the external pH decreases below 4.0 (Foster and Hall, 1991). Twelve proteins are induced and six repressed during the pre-shock adaptation (Foster and Hall, 1990) and the identity of a number of the induced proteins has been determined. They are a Mg²⁺-dependent proton-translocating ATPase, an Fe²⁺-binding regulatory protein (Foster, 1991; Foster and Hall, 1991) and DNA polymerase I (Foster and Bearson, 1994). The post-shock phase involves the induction of forty-three acid shock proteins which are essential in the prevention or repair of cellular acid damage (Foster, 1993).
The pH homeostasis system that is induced by pre-shock adaptation enables *S. typhimurium* to synthesise the acid shock proteins when the external pH falls below 4.5. Protein synthesis is very sensitive to intracellular pH values and does not function well at an internal pH less than 6.0 (Foster, 1993). When the extracellular pH is 3.3, the internal pH of unadapted cells is 4.5 (Foster and Hall, 1991). However, pre-shock adapted cells are able to maintain an internal pH nearer 6.0 when the external environment is acidic (pH 3.3) (Foster and Hall, 1991). As a result, pre-shocked cells, but not unadapted cells, can produce the protective acid shock proteins during severe acid exposure.

Foster (1993) has demonstrated that of the forty-three acid shock proteins, twenty-two were induced by decreasing internal pH. Thirteen others were induced in response to an external pH of or less than 4.5. The inducing signal for the eight remaining acid shock proteins was not determined. Thus, there are at least two different classes of acid shock protein genes and regulatory systems which are based upon a response to internal or external pH.

Both pre- and post-acid shock stages of the ATR are required for maximum protection against low pH (Foster, 1993). However, either stage will confer some level of protection against a lethal pH. A single, brief period (fifteen minutes) of acid shock at pH 4.3 is sufficient to allow *S. typhimurium* to tolerate a challenge pH of 3.3. It was found that acid shock exposure of more than thirty minutes did not afford this protection (Foster, 1993). Thus, a subset of acid shock proteins are synthesised transiently at pH 4.3 which then disappear, leaving the cell defenceless against extreme acid stress.

The acid-adaptation responses described above are summarised in Fig. 1.3. All three responses are induced in *S. typhimurium* cells that are growing exponentially (log phase ATR). However, two additional systems of acid resistance can occur in stationary phase *S. typhimurium* cells (Lee *et al.*, 1994). One is a pH-dependent system (stationary phase ATR) which provides a higher level of acid resistance than the log phase ATR. Stationary phase ATR requires a long period (two hours) of exposure to low pH for full induction (Lee *et al.*, 1994). Maximal induction takes place at an external pH of 4.3 and involves the synthesis of just fifteen acid shock proteins. The other acid adaptation system is not induced by low pH and appears to be part of a general stress resistance mechanism that is induced in the stationary phase (Lee *et al.*, 1994).
Fig. 1.3. The Acid Tolerance Response (ATR) of *Salmonella typhimurium*.

**Unadapted**

Unadapted

\[
\text{pH}_{7.6} \rightarrow \text{pH}_{3.3} \quad \text{(pH}_{4.5}\text{)} \quad \text{DEATH}
\]

**Acid Shock**

Acid Shock

\[
\text{pH}_{7.6} \rightarrow \text{pH}_{4.3} \rightarrow \text{pH}_{3.3} \quad \text{(pH}_{4.7}\text{)} \quad \text{SURVIVAL}
\]

ASP synthesis

**Pre-shock**

Pre-shock

\[
\text{pH}_{7.6} \rightarrow \text{pH}_{5.8} \rightarrow \text{pH}_{3.3} \quad \text{(pH}_{5.1}\text{)} \quad \text{SURVIVAL}
\]

ASP synthesis

**Pre- and Post-shock**

Pre- and Post-shock

\[
\text{pH}_{7.6} \rightarrow \text{pH}_{5.8} \rightarrow \text{pH}_{4.3} \rightarrow \text{pH}_{3.3} \quad \text{(pH}_{5.1}\text{)} \quad \text{SURVIVAL}
\]

ASP synthesis

Adapted from Foster (1993).

\[\text{pH}: \text{intracellular pH.} \quad \text{pH}_{o}: \text{extracellular pH.} \quad \text{ASP}: \text{Acid shock protein.}\]
1.12. Aims of the Study.

The purpose of this work was to investigate oxygen-independent tuberculocidal mechanisms of macrophages (section 1.9.). To this end, attempts were made to activate human macrophages *in vitro* for antimycobacterial activity. Also, a passaged strain of *M. tuberculosis* was tested for susceptibility to toxic agents (section 1.10.). A protocol for activating guinea pig macrophages to kill *M. tuberculosis in vitro* has already been elucidated (O’Brien and Andrew, 1991; O’Brien et al, 1991). The guinea pig model was therefore extensively used in experiments. Three potential macrophage tuberculocidal mechanisms were studied.

1. Lysosomal Enzymes.

Lysosomes were isolated from activated and non-activated guinea pig macrophages. The enzymes were tested for activity against *M. tuberculosis*.

2. Reactive Nitrogen Intermediates (RNI).

The toxicity of RNI for strains of *M. tuberculosis* was assessed and the ability of murine, human and guinea pig macrophages to produce RNI was determined. An inhibitor of RNI synthesis was tested for its ability to reverse the killing of *M. tuberculosis* by activated guinea pig macrophages.

3. Toxic Aminoaldehydes.

Ornithine decarboxylase (ODC) is the first enzyme in a pathway that leads to the production of toxic aminoaldehydes. The ODC content of activated and non-activated guinea pig macrophages was therefore assessed.

In addition, the response of mycobacteria to acid stress was investigated. *Mycobacterium smegmatis mc²155* was exposed to mildly acidic conditions and was subsequently tested for the ability to survive at extreme acidity. The purpose was to demonstrate an acidification tolerance response in a mycobacterium.
2. MATERIALS AND METHODS.
2.1. Chemicals.

All chemicals were obtained from Sigma Chemical Company Ltd. (U.K.) or BDH Laboratory Supplies (U.K.), unless otherwise stated in the text.

2.2. Tissue Culture Reagents.

All tissue culture reagents were obtained from Gibco BRL (U.K.), except where stated. Hanks balanced salt solution, without phenol red, was buffered with 25mM Hepes, pH 7.4 (HBSS-Hepes). Foetal and newborn calf sera were stored frozen at -20°C. Before experimental use they were heat inactivated at 56°C for 30 minutes (HIFCS, HINCS).

RPMI 1640 tissue culture medium was supplemented with 1mM L-glutamine and, where appropriate, 10% (v/v) HIFCS or HINCS. Serumless tissue culture medium (Neuman-Tytell) was also supplemented with 1mM L-glutamine. All tissue culture media were stored at 4°C until required.

Recombinant human interferon-γ (IFN-γ) (a gift from G.R. Adolf, Boehringer Ingelheim, Austria) was stored at -70°C at a specific activity of 1x10^6 U ml^{-1}. Recombinant murine IFN-γ was also stored at -70°C at a specific activity of 1x10^6 U ml^{-1}.

2.3. Tissue Culture Plasticware.

All tissue culture flasks and plates were obtained from Nunc (U.K.). Syringes and needles were obtained from Becton Dickinson (U.K.) and Sherwood Medical (U.K.) respectively.

2.4. Growth and Maintenance of Cell Lines.


The U937 cells were recovered from stocks held in liquid nitrogen in the Department of Microbiology and Immunology, University of Leicester. U937s were grown in RPMI 1640
medium plus HIFCS at 37°C and 5% (v/v) CO₂. The cell line was maintained by subculturing 5ml of the U937 suspension into 10ml of fresh medium every two days. Two days before the start of an experiment, 10ml of the U937 suspension were subcultured into 20ml of medium. The medium was then changed after 24 hours. This procedure allowed a larger number of U937s to be harvested for the experiment. When activated monocytes were required, 100U ml⁻¹ of recombinant human IFN-γ were included in the medium. Fresh IFN-γ was added when the medium was changed after the first 24 hour incubation.

When required for experimental purposes, the U937s were harvested by centrifugation at 160g for 10 minutes. They were then resuspended in HBSS-Hepes before being counted microscopically with a haemocytometer. Viability was assessed by trypan blue exclusion (section 2.4.3.).


The J774 cells were obtained from the European Collection of Animal Cell Cultures (U.K.). J774s were grown in RPMI 1640 medium plus HIFCS at 37°C and 5% (v/v) CO₂. The J774s were subcultured when a confluent monolayer formed, which was typically after four days. The old medium was removed and 5ml of trypsin-EDTA solution were added to the monolayer. The trypsin-EDTA solution consisted of 0.05% (w/v) trypsin and 0.02% (w/v) EDTA in Ca²⁺- and Mg²⁺-free Hanks balanced salt solution, without phenol red. The cells were incubated for 5 minutes at 37°C and 5% (v/v) CO₂, after which the trypsin-EDTA solution was poured off and the flasks reincubated at 37°C and 5% (v/v) CO₂ for 10 minutes. 10ml of RPMI 1640 medium plus HIFCS were added to the monolayer and the cells scraped off the surface of the flask with a transfer pipette. 1ml of cells was then transferred into a new tissue culture flask which contained 9ml RPMI 1640 medium plus HIFCS.

When required for experimental purposes, the J774 cells were removed from the tissue culture flask and were counted microscopically with a haemocytometer. Viability was determined by trypan blue exclusion, as described in section 2.4.3. The concentration was adjusted to 1.28x10⁵ cells ml⁻¹ RPMI 1640 plus HIFCS. 500μl of the J774 suspension were added to the wells of 24 well tissue culture plates. The cells were incubated at 37°C and 5% (v/v) CO₂ for 24
hours to allow adherence. The next day the monolayers were washed once in HBSS-Hepes and 500μl of fresh medium was added. In order to activate the cells, 50U of recombinant murine IFN-γ were added to each well. The macrophages were reincubated and fresh medium, with or without IFN-γ, was added after 24 hours.

2.4.3. Trypan Blue Exclusion Assay.

20μl of the cell suspension to be counted were mixed with an equal volume of 0.2% (v/v) trypan blue in Hanks balanced salt solution, without phenol red. 20μl of this mixture were then applied to the haemocytometer and the cells counted. Viable cells were white in colour whereas non-viable cells stained dark blue.

2.4.4. Preservation of Cell Lines in Liquid Nitrogen.

In case of contamination or death of the cells, reserves of U937s and J774s were held in liquid nitrogen.

U937s were allowed to grow until they reached a dense culture and were then harvested as described (section 2.4.1.). J774s were grown to confluence before being removed from the surface of the flask as described (section 2.4.2.). The number of viable cells was counted with a haemocytometer (section 2.4.3.) and the concentration adjusted to 1x10^7 cells ml⁻¹ in RPMI 1640 plus 20% (v/v) HIFCS and 20% (v/v) glycerol. The cell suspension was aliquoted as 1ml volumes into sterile cryotubes and these were placed inside a cryo 1°C freezing container (Nalgene, U.K.) containing isopropanol. The container was then kept at -70°C to allow the cell suspensions to freeze. The length of time the cells stayed in the freezer was based on the instructions supplied by Nalgene. Once frozen, the cryotubes were removed and transferred to liquid nitrogen for storage.

2.4.5. Recovery of Cell Lines from Liquid Nitrogen.

The cells were thawed in a 37°C water bath and were added to 9ml of RPMI 1640 plus HIFCS in the appropriate tissue culture flask. After two to four days the cells had resumed a normal growth rate.
2.5. Microorganisms.

Eight strains of *M. tuberculosis* (H37RaHR, H37Ra, B1453, 79112, 79500, H37Rv, 12646 and 79499) and two of *M. bovis* (81470 and BCG) were cultured and maintained for experimental use. *Mycobacterium tuberculosis* H37Ra was obtained from the National Collection of Type Cultures (U.K.). *Mycobacterium bovis* BCG was received as a freeze-dried culture (Evans Medical Ltd., U.K.). All the other strains were obtained from the Leicester University Culture Collection. They were recovered from frozen cultures which had been stored at -20°C on Lowenstein-Jensen slopes. *Mycobacterium bovis* 81470 had originally been a clinical isolate from the Leicester Royal Infirmary.

The origins and properties of the *M. tuberculosis* strains have been described previously (Jackett *et al*, 1978). The virulence of the strains in guinea pigs, known as the root index of virulence (RIV), has been reported by Jackett *et al* (1978) and Mitchison *et al* (1963). RIV is a measurement of the rate at which pathological lesions develop in the organs *in vivo* following an intramuscular challenge. The RIV of the tuberculous strains are shown in Table 2.1.

Mouse-passaged *M. tuberculosis* H37Rv was kindly donated by Dr D.B. Lowrie (National Institute for Medical Research, London). The H37Rv had been passaged once through the mouse by Dr M.J. Colston (National Institute for Medical Research, London). The initial dose was 1x10⁶ bacilli and it was administered as an intraperitoneal injection. *Mycobacterium tuberculosis* H37Rv was recovered from the spleen after twenty-eight days. The original strain that had been used to infect the mouse was also supplied for comparison in viability tests (section 2.18.).

*Mycobacterium tuberculosis* strain 24, strain 24 containing plasmid p16R1 and strain 24 containing plasmid pYZ66 were generously supplied by Dr Y. Zhang (St. Mary's Hospital Medical School, London). pYZ66 was a derivative of p16R1 and carried the *M. tuberculosis* katG gene (Zhang *et al*, 1993). Both plasmids contained the hygromycin resistance gene as a selective marker.

*Mycobacterium smegmatis* mc²155 (Snapper *et al*, 1990) was kindly donated by Dr W.R. Jacobs (Albert Einstein College of Medicine, New York).
Table 2.1. *Mycobacterium tuberculosis* Strain Characteristics.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Origin</th>
<th>RIV&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Catalase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37RaHR</td>
<td>Mutant of H37Ra</td>
<td>0.11</td>
<td>-</td>
</tr>
<tr>
<td>H37Ra</td>
<td>NCTC 7417</td>
<td>0.52</td>
<td>+</td>
</tr>
<tr>
<td>B1453</td>
<td>British patient</td>
<td>0.77</td>
<td>-</td>
</tr>
<tr>
<td>79112</td>
<td>Indian patient</td>
<td>0.92</td>
<td>+</td>
</tr>
<tr>
<td>79500</td>
<td>Indian patient</td>
<td>0.98</td>
<td>+</td>
</tr>
<tr>
<td>H37Rv</td>
<td>NCTC 7416</td>
<td>1.01</td>
<td>+</td>
</tr>
<tr>
<td>12646</td>
<td>British patient</td>
<td>1.17</td>
<td>+</td>
</tr>
<tr>
<td>79499</td>
<td>Indian patient</td>
<td>1.29</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains showing RIVs in guinea pigs of ≥ 1.0 and ≤ 1.0 were termed high- and low-virulence strains respectively.
2.6. Growth and Maintenance of Bacterial Cultures.

The mycobacteria were maintained in 10ml volumes of Middlebrook 7H9 broth (Difco Laboratories, MI) supplemented with 10% (v/v) Albumin Dextrose Complex (ADC, Difco) at 37°C and 5% (v/v) CO₂. 25μg ml⁻¹ hygromycin were added to cultures of *M. tuberculosis* strain 24 (p16R1) and 24 (pYZ86) to ensure the plasmid was maintained in the bacteria. The strains of *M. tuberculosis* and *M. bovis* were subcultured every seven days and were kept in static incubators. *Mycobacterium bovis* BCG was also grown at 37°C and 5% (v/v) CO₂ in 50ml volumes of freshly-made liquid Kirchner medium for use in one experiment (section 2.14.7). Kirchner medium consisted of 19g l⁻¹ disodium hydrogen phosphate, 2g l⁻¹ potassium dihydrogen phosphate, 0.5g l⁻¹ magnesium sulphate, 2.5g l⁻¹ trisodium citrate, 5g l⁻¹ L-asparagine, 2% (v/v) glycerol and 0.12% (v/v) phenol red (Mitchison *et al.*, 1983). The pH was in the range 6.9-7.2 and the medium was filter sterilised before use. *Mycobacterium bovis* BCG was subcultured every two weeks when grown in Kirchner medium.

*Mycobacterium smegmatis* mc²155 was initially cultured in ADC-supplemented 7H9 broth from the fresh growth on the Lowenstein-Jensen slope supplied by Dr W.R. Jacobs. After being incubated overnight, at 37°C and with shaking (200 r.p.m.), mc²155 was streaked onto 7H11 agar (Difco) supplemented with 10% (v/v) Oleic acid Albumin Dextrose Complex (OADC, Difco). The plates were incubated for 48 hours at 37°C and were then stored at 4°C. To maintain the culture at 4°C, colonies were picked every two weeks and streaked onto a new 7H11 plate. When required for experimental purposes, colonies of mc²155 were cultured overnight, at 37°C and with shaking, in 7H9 broth supplemented with ADC.

2.6.1. Preservation of Mycobacterial Strains in Glycerol.

Tubercle strains were grown in 10ml aliquots of 7H9 plus ADC for seven days (section 2.6.). *Mycobacterium smegmatis* mc²155 was grown overnight in 10ml of ADC-supplemented 7H9 (section 2.6.). 4.5ml of 50% (v/v) glycerol in 7H9 were then added to each culture, resulting in a 15% (v/v) glycerol solution. The culture was gently mixed and 1ml volumes were aliquoted into sterile eppendorf tubes which were subsequently stored at -70°C.
2.7. Preparation of Mycobacteria.

Seven day old cultures were used in each experiment. Prior to use, the mycobacteria were harvested by centrifugation at 2000g for 10 minutes and were washed twice by centrifugation in 0.01% (v/v) Tween 80 in nanopure water. The pellet was resuspended in the appropriate medium and sonicated for three 5 second bursts at 40W (Ultrasonic Engineering, U.K.) to disrupt bacterial clumps. The mycobacteria were counted by microscopy using a Thoma chamber and were then diluted to the appropriate working concentration.

2.8. Viable counting of Mycobacteria.

50μl of the sonicated mycobacterial suspension were added to 450μl of sterile nanopure water. This was serially diluted to a 10^-4 dilution. 50μl volumes of the 10^-2, 10^-3 and 10^-4 dilution were pipetted onto one third of a Middlebrook 7H11 agar plate with OADC enrichment. After the samples had dried onto the agar, the plates were wrapped in plastic bags and incubated at 37°C until individual colonies were visible. Typically, this took approximately three weeks. In the case of M. smegmatis mc²155, incubation was for 48 hours.

The number of colonies at a particular dilution was counted with a colony counter (Afterman and Co. Ltd., U.K.). At least one hundred colonies were counted. The number of viable mycobacteria in the original suspension was then calculated.

2.9. Animals.

Female Dunkin Hartley guinea pigs, weighing approximately 300-400g each, were obtained from David Hall Ltd. (U.K.). The animals were housed and maintained in the Biomedical Services Unit at Leicester University.

2.10. Vaccination of Guinea Pigs.

Mycobacterium bovis BCG was prepared in 100μl HBSS-Hepes for each injection, as
described (section 2.7.). Initially, guinea pigs were intraperitoneally vaccinated with \(2 \times 10^7 M. bovis\) BCG. Three weeks later, they were intramuscularly boosted in the thigh with \(1 \times 10^7\) BCG. Eight days after the booster injection, the guinea pigs were intravenously challenged via an ear vein with \(1 \times 10^7\) BCG. Six days later, they were killed either by dislocation of the neck followed by exsanguination or by intraperitoneal injection of 8ml pentabarbitone (Animal Care Ltd., U.K.). Guinea pigs given this vaccination schedule were termed vaccinated/boosted/challenged (VBC).

This vaccination protocol has been reported to result in activated alveolar macrophages capable of killing \(M. tuberculosis\) in vitro (O'Brien and Andrew, 1991; O'Brien et al, 1991).

2.11. Collection of Alveolar Macrophages.

For each guinea pig, three 20ml volumes of RPMI 1640 medium plus 5U ml\(^{-1}\) of heparin (Leo Laboratories Ltd., U.K.) were prepared in 20ml syringes and kept on ice. Alveolar macrophages were collected by one of two methods.  

Method 1: The lungs and trachea were removed intact. Using Spencer-Wells forceps, the trachea was clamped and the lungs suspended from a retort stand via the forceps. The outside of the lungs was washed with HBSS-Hepes. The lungs were lavaged, via the trachea, by injection of 20ml RPMI 1640 plus heparin. The trachea was then cut below the forceps and the lavage fluid poured out of the lungs into universals held on ice. The lungs were resuspended from the retort stand and the lavage procedure repeated twice more. The recovered medium was centrifuged at 200g for ten minutes to harvest the macrophages. The cells were then washed once in HBSS-Hepes by centrifugation and were resuspended in the appropriate medium. The number of macrophages was estimated microscopically with a haemocytometer. Viability was assessed by trypan blue exclusion (section 2.4.3.).

Method 2: This method was used when sterile macrophages were required. The lungs were exposed and the top of the trachea clamped. 20ml of lavage fluid were injected through the trachea into the lungs. Without removing the needle, the lungs were carefully massaged and then the lavage fluid was drawn back into the syringe. The trachea was clamped lower down and the lavage repeated
with fresh medium. The three washings were collected into sterile universals on ice and the macrophages were harvested, washed and counted as above.


A respiratory burst was detected by measuring the release of hydrogen peroxide ($H_2O_2$) from cells. Production of $H_2O_2$ was assayed fluorimetrically, as described previously (Jackett et al., 1981b).

Before the start of the assay, cells were washed with HBSS-Hepes to remove any residual RPMI 1640 medium. This was necessary as the phenol red present in RPMI 1640 interferes with the assay for $H_2O_2$. Adherent cell monolayers were washed twice with 1 ml HBSS-Hepes. U937s were washed twice by centrifugation at 160g for 10 minutes.

The substrate, p-hydroxyphenyl acetic acid (POHPAA), was freshly prepared at a concentration of 7.4mg ml$^{-1}$ in HBSS-Hepes. The enzyme, horseradish peroxidase (HRP), was diluted ten-fold from a stock concentration of 100U ml$^{-1}$ in HBSS-Hepes. Both substrate and enzyme were pre-warmed for 5 minutes at 37°C. 50μl of POHPAA and 50μl of HRP were then added to 1×10$^6$ cells in 800μl HBSS-Hepes.

When required, the cell stimulus, phorbol myristate acetate (PMA), was diluted from a stock concentration of 1mg ml$^{-1}$ in DMSO to 10μg ml$^{-1}$ in HBSS-Hepes. 100μl of PMA were added to the cells to give a 1ml final volume. 100μl HBSS-Hepes were added to the control cells. Alternatively mycobacteria were used as a cell stimulus. The bacteria were prepared as described (section 2.7.) and were resuspended at a concentration of 1×10$^9$ ml$^{-1}$ HBSS-Hepes. POHPAA and HRP were added, as before, to 1×10$^6$ cells in 800μl HBSS-Hepes. 1×10$^8$ mycobacteria were then added or, for the control cells, 100μl HBSS-Hepes.

Cells were incubated for 60 minutes at 37°C and 5% (v/v) CO$_2$. After the incubation period, the supernatants were removed from cell monolayers and reserved. Samples containing U937s were centrifuged at 160g for 10 minutes and then the supernatants were removed and reserved. The reaction was stopped by the addition of 1ml ice cold 0.1M borate buffer, pH10.4, to the supernatants. The samples were incubated in the dark at room temperature for 10 minutes.
The fluorescence of the samples was then measured at an excitation wavelength of 313nm and an analysis wavelength of 414nm using an LS-2B Filter Fluorimeter (Perkin-Elmer, U.K.). When virulent mycobacteria had been used as the stimulus, the supernatants were filtered through 0.22µm filters (Millipore, MA) prior to measuring the fluorescence.


H$_2$O$_2$ was diluted to 200µM in HBSS-Hepes. Amounts of H$_2$O$_2$, ranging from 0-100nmoles, were prepared in duplicate. The final volume was adjusted to 900µl with HBSS-Hepes. 50µl of both POHPAA and HRP, as used for the cellular assay, were then added and the standards incubated simultaneously with the cells. The reaction was stopped with 1ml 0.1M ice cold borate buffer, pH10.4, and, after the 10 minute incubation in the dark, the fluorescence was measured as above. A calibration curve was constructed by plotting fluorescence against the concentration of H$_2$O$_2$.

A H$_2$O$_2$ standard assay was included in every experiment that measured cellular H$_2$O$_2$ release. The concentration of H$_2$O$_2$ produced by the cells was then calculated from the corresponding calibration curve.

2.13. Measuring Cellular DNA.

Determination of cellular DNA content was based on an assay previously described by Labarca and Paigen (1980).

Monolayers were washed six times in 500µl HBSS-Hepes to remove any residual tissue culture medium. If the cells had been infected with mycobacteria, 150µl of ethanol were added to 350µl HBSS-Hepes after the last wash to ensure fixation of the bacilli. The cells were scraped off the surface of the well with a plunger from a 1ml syringe and were broken open by three 5 second bursts from the sonicator. The resulting suspensions were stored frozen at -20°C.

When required, the thawed cell samples were resuspended by vigorous agitation. Hoechst 33258 (2'-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimidazole), which was stored at a stock concentration of 2.5mg ml$^{-1}$ in DMSO, was diluted five hundred-fold.
in 0.05M phosphate buffer, pH7.4, containing 2M sodium chloride. 250μl of the Hoechst solution and 500μl of 0.1M phosphate buffer, pH7.4, containing 4M sodium chloride were added to the cell samples. After overnight incubation at room temperature, the fluorescence of the samples was measured at an excitation wavelength of 358nm and an analysis wavelength of 414nm using an LS-2B Filter Fluorimeter (Perkin-Elmer, U.K.).

2.13.1. DNA Standard Curve.

Calf thymus DNA was stored at a concentration of 200μg ml⁻¹ in 5mM sodium hydroxide. When required, it was diluted ten-fold in HBSS-Hepes. A range of DNA concentrations, from 0-10μg ml⁻¹, were prepared in duplicate in 500μl HBSS-Hepes. To these were added 250μl of the Hoechst solution and 500μl of 0.1M phosphate buffer, pH7.4, containing 4M sodium chloride. After overnight incubation at room temperature, the fluorescence was measured as described above.


These experiments were based on the work published by Denis (1991b) which reported that human monocytes could be activated to kill M. tuberculosis in vitro. The method followed, as closely as possible, that of Denis (1991b) as it was desirable to reproduce these results.

2.14.2. Isolation and Culture of Human Peripheral Blood Monocytes.

50ml of venous blood were collected from healthy volunteers who had been vaccinated in the past with M. bovis BCG. 10ml of CPD-adenine (Baxter Health Care Ltd., U.K.) were added to the blood to prevent clotting. The mixture was gently agitated, then 15ml aliquots of blood were layered onto 10ml of Ficoll-Paque (Pharmacia, Sweden) in universals. Monocytes were separated from other cells by centrifugation at 500g for 30 minutes.
After centrifugation, the plasma was removed and retained as a source of autologous serum. 200U of thrombin (Armour Pharmaceutical Company, IL) were added to approximately 10ml of plasma and, after the fibrin had clotted, the liquid serum was collected and stored on ice. Long term storage was at -20°C.

The layer of monocytes, which occurred between the plasma and Ficoll-Paque, was removed. 20ml of HBSS-Hepes were added and the monocytes were washed by centrifugation at 160g for 10 minutes. This washing step was repeated and the cell pellets were resuspended in a small amount of HBSS-Hepes, pooled and counted microscopically with a haemocytometer. Viability was assessed by trypan blue exclusion (section 2.4.3.).

The monocytes were plated onto glass coverslips (Richardsons, U.K.) in 24 well tissue culture plates at 1x10⁶ cells well⁻¹ in 1ml RPMI 1640 medium plus 10% (v/v) autologous serum. The plates were incubated for 1 hour at 37°C and 5% (v/v) CO₂ to allow the monocytes to adhere to the coverslips. After this time, the monolayers were washed twice with RPMI 1640 plus 10% (v/v) autologous serum to remove the non-adherent cells. After the second wash, 1ml of RPMI 1640 plus 5% (v/v) autologous serum was added to each well.

2.14.3. Immunomodulators.

A combination of interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and 1-α, 25-dihydroxycholecalciferol (calcitriol) was used to activate the monocytes (Denis, 1991b). Recombinant human IFN-γ was a gift from G.R. Adol, Boehringer Ingelheim (Austria). It was stored at -70°C at a specific activity of 1x10⁶U ml⁻¹. Recombinant human TNF-α was stored at -20°C at a specific activity of 2x10⁵U ml⁻¹. Calcitriol was a gift from Roche (U.K.). It was dissolved in ethanol and was stored as a 1mM solution in the dark and under an atmosphere of nitrogen at -20°C.

1000U IFN-γ ml⁻¹, 100U TNF-α ml⁻¹ and 10⁻⁹M calcitriol (Denis, 1991b) were added to half of the wells. The monolayers were then incubated overnight in the presence or absence of the immunomodulators.
2.14.4. Infection of the Monocytes.

Monolayers were infected after the overnight incubation. *Mycobacterium tuberculosis* H37Rv was prepared as described (section 2.7.) at a concentration of $1 \times 10^8 \text{ ml}^{-1}$ RPMI 1640 plus 5% (v/v) autologous serum. 100μl aliquots were added to each well, resulting in a 10:1 ratio of mycobacteria to monocytes. Immunomodulators were present during the infection. The plates were incubated, at 37°C and 5% (v/v) CO₂, for 6 hours to allow phagocytosis. The monolayers were then washed six times with warm RPMI 1640 plus 5% (v/v) autologous serum to remove any remaining extracellular mycobacteria. After the last wash, 1 ml of RPMI 1640 plus 5% (v/v) autologous serum was added to each well. The monocytes were reincubated, until the appropriate time, at 37°C and 5% (v/v) CO₂.

2.14.5. Assessment of Antimycobacterial Activity.

At each time point (0, 4 and 8 days) the supernatant was removed from the appropriate wells, reserved, and replaced with 1 ml HBSS-Hepes. 1 ml 0.25% (w/v) SDS in HBSS-Hepes was added to each well to lyse the cells. The monolayers were left at room temperature for 5 minutes and then both the supernatant and the monocytes were sonicated (Ultrasonic Engineering, U.K.) for three periods of 5 seconds ensuring the liberation of intracellular bacteria and the disruption of any mycobacterial clumps that had formed during incubation. Viable count assays (section 2.8.) were then used to estimate the number of intracellular and extracellular *M. tuberculosis* H37Rv in each well.

In other wells, the monocytes were fixed with glutaraldehyde (Agar Aids, U.K.). The supernatant was removed and the monolayer washed four times in HBSS-Hepes after which the well was filled with 2.5% (v/v) glutaraldehyde. The monolayers were left for at least 10 minutes at room temperature. The coverslips were then stained in their wells by the cold Ziehl-Neelsen method. The monolayers were stained with Fuschine RAL 22 cold AFB stain (BDH, U.K.) for 1 minute, washed four times with distilled water, covered with 25% (v/v) sulphuric acid for 1 minute and counterstained for 30 seconds with 0.2% (v/v) methylene blue (personal communication from J. Warwick-Davies, National Institute of Medical Research, London). The numbers of intracellular mycobacteria were counted microscopically in at least one hundred
infected cells.

In addition the number of monocytes in the monolayers was tested at each time point by measuring the cellular DNA content of certain wells (section 2.13.).


As an indication of whether the monocytes were being activated by the immunomodulators, production of H$_2$O$_2$ was assayed. Monocytes were isolated and cultured as described above (section 2.14.2.) and were incubated either in the presence or absence of immunomodulators overnight (section 2.14.3.). H$_2$O$_2$ release, on stimulation with either M. tuberculosis H37Rv or PMA, was then tested as described (section 2.12.).


These experiments were based on those reported by Crowle and May (1981) in which a technique was developed to activate human macrophages to control the growth of tubercle bacilli in vitro. The methods were closely based on those published, in order to reproduce the results.


An antigen, derived from M. bovis BCG, was used to stimulate lymphocytes to produce cytokines which were for later use (section 2.14.12.). The antigen was produced by following the method described by Crowle (1972).

*Mycobacterium bovis* BCG was grown, over a period of 4 weeks, in 50ml volumes of Kirchner medium (section 2.6.) at 37°C and 5% (v/v) CO$_2$. The mycobacteria were harvested by centrifugation at 2000g for 10 minutes and were then washed three times in distilled water by centrifugation. The pellet was resuspended in 5ml of distilled water and was vigorously agitated for 2 minutes to produce a fine suspension of bacilli.

The mycobacterial suspension was added to 400ml of freezing (-20°C) acetone to extract the lipids in the bacterial wall. The mycobacteria were left in the acetone for 20 minutes, with continuous stirring, at 4°C. The bacteria were retrieved by filtering the mixture through a Buchner funnel, the filter paper was then left at 4°C for 30 minutes to allow the bacilli to dry.
The dried bacteria were weighed and were resuspended in 100 volumes of 1% (w/v) sodium hydrogen carbonate. The mixture was vigorously agitated for 2 minutes to ensure the bacilli were finely dispersed. The suspension was then sterilised by autoclaving for 15 minutes at 121°C after which it was cooled to 37°C. A sterile solution of trypsin (Bovine Pancreas type III) was added to the autoclaved bacteria to make a 0.005% (w/v) solution in sodium hydrogen carbonate. The bacilli were left to be digested for 24 hours at 37°C. After this time, more trypsin was added to make a 0.0025% (w/v) solution in sodium hydrogen carbonate. The mycobacteria were then incubated for a further 2 hours at 37°C.

The digested bacteria were pelleted by centrifugation at 2000g for 10 minutes and the supernatant was filtered through a 0.2μm Acrodisc (Gelman Sciences, MI) to remove any remaining bacilli. The filtrate was concentrated five-fold by partial lyophilisation and was then dialysed against distilled water for 2 hours at 4°C. The filtrate was then completely lyophilised to produce the TE antigen which is a very light, fluffy, white powder.

TE antigen was dissolved in distilled water at a concentration of 10mg ml⁻¹. The resulting solution was sterilised through a 0.2μm Acrodisc and was stored at -20°C.


Peripheral blood monocytes were isolated as described (section 2.14.2.). The cells were plated at 5x10⁶ monocytes well⁻¹ in 1ml of RPMI 1640 plus 1% (v/v) autologous serum in 24 well tissue culture plates and were incubated for an hour, at 37°C and 5% (v/v) CO₂, to allow adherence to the plastic. After this time, the monocytes were washed twice with warm RPMI 1640 plus 1% (v/v) autologous serum and the washings were kept as a source of non-adherent lymphocytes. 1ml of serumless medium plus 1% (v/v) autologous serum was then added to each well and the monocytes were incubated for 7 days, at 37°C and 5% (v/v) CO₂, to allow the monocytes to mature into macrophages. During this time the medium above each monolayer was not changed.


The non-adherent lymphocytes (section 2.14.9.) were used to produce a supernatant
(GIF) that contained an unknown combination of cytokines that had been reported to activate homologous macrophages to control a mycobacterial infection (Crowle and May, 1981).

The washings were distributed in equal volume between five universals. TE antigen (section 2.14.8.) was added to each universal at a concentration of 100μg ml⁻¹. The lymphocytes were incubated for 3 hours at 37°C and 5% (v/v) CO₂, after which the majority of the medium was discarded, by gentle pipetting, leaving 2ml of medium in the bottom of each universal. The lymphocytes were then incubated for a further 72 hours, at 37°C and 5% (v/v) CO₂.

After this time, the contents of each universal were pooled and the medium was filtered through a 0.2μm Acrodisc to remove the lymphocytes. The resulting GIF was aliquoted as 1ml volumes and was stored at -70°C for later use.

2.14.11. Infection of the Macrophages.

After the 7 day incubation period, the newly matured macrophages were infected with \textit{M. bovis} BCG. The bacteria were prepared, as described in section 2.7., at a concentration of 25x10⁶ bacilli ml⁻¹ serumless medium plus 5% (v/v) autologous serum. The medium was removed from each well and was replaced with 1ml of serumless medium plus 5% autologous serum containing the mycobacteria. The infection ratio was thus five bacilli to one macrophage.

The monolayers were incubated for an hour, at 37°C and 5% (v/v) CO₂, to allow phagocytosis. After this time, they were washed six times in warm serumless medium plus 1% (v/v) autologous serum to remove the extracellular bacteria (time zero). The culture period was then continued, at 37°C and 5% (v/v) CO₂, for a further 7 days in 1ml of serumless medium plus 1% (v/v) autologous serum. During this second 7 day incubation the medium was not changed.


Half of the macrophage monolayers were given a 100μl aliquot of GIF 30 minutes, 2 days and 4 days post-infection. To the control wells, 100μl of RPMI 1640 plus 1% (v/v) autologous serum were added.

Certain assays were performed immediately after the infection period (time zero). Three monolayers were tested for the numbers of phagocytosed *M. bovis* BCG. The supernatant was removed and 500μl of a saturated solution of digitonin (8mg ml\(^{-1}\) HBSS-Hepes) were added to each monolayer to lyse the cells. The macrophages were allowed to stand at room temperature for 5 minutes after which they were sonicated (Ultrasonic Engineering, U.K.) for three 5 second bursts to disrupt mycobacterial clumps. Viable count assays (section 2.8.) were then used to ascertain the number of intracellular mycobacteria in each monolayer at the start of the 7 day infection period. Cellular DNA assays (section 2.13.) were also performed on three monolayers to estimate the number of macrophages at time zero.

After the 7 day incubation, the remaining wells were investigated for any effect on the growth of the mycobacteria. The supernatant was removed from each monolayer and was reserved. The macrophages were washed twice in HBSS-Hepes, the washings being pooled with the supernatant. 500μl of a saturated solution of digitonin were added to each well and to each supernatant. They were left to stand at room temperature for 5 minutes and were subsequently sonicated for three 5 second bursts. Viable count assays (section 2.8.) were then used to estimate the number of intracellular and extracellular mycobacteria in each well. In addition, cellular DNA assays (section 2.13.) were performed on certain monolayers to monitor the change in macrophage number over the 7 day incubation period.

2.15. Investigating Lysosomes for Antimycobacterial Activity.

This work was a continuation of that begun by O’Brien (1992). Some of the results have been reported before (O’Brien, 1992).

2.15.1. Preparation of Macrophage Homogenates.

Alveolar macrophages from vaccinated and non-vaccinated guinea pigs (section 2.10.) were collected using method 1 (section 2.11.). After being harvested, the cells were resuspended in 5ml 2.5M sucrose, 1mM EDTA, 0.1% (v/v) ethanol and 5U ml\(^{-1}\) heparin (Leo Laboratories,
U.K.), pH 7.4 (SVEH). The macrophages were counted (section 2.4.3.), washed in SVEH by centrifugation at 600g for ten minutes and resuspended in 2ml SVEH. The cells were broken by passage through a 25 gauge needle. After every three passages, the suspension was centrifuged at 800g for ten minutes to sediment the nuclei, erythrocytes and unbroken cells. The resulting post-nuclear supernatant (PNS) was retained and the intact macrophages resuspended in 2ml SVEH. The macrophages were passaged until at least 90% breakage was achieved; typically this required nine passages. Breakage was regularly assessed with a haemocytometer. The reserved supernatants were pooled. Part of the PNS was retained for enzyme assays (section 2.15.3.) and part for a hydrogen peroxide assay (section 2.12.), the remainder was applied to a sucrose gradient.

2.15.2. Subcellular Fractionation of Macrophage Homogenates.

These methods were based on those described previously by Segal and Peters (1977) and Lowrie et al (1979).

Analytical isopycnic ultracentrifugation of PNS was performed using a linear, continuous sucrose gradient. The discontinuous 30ml gradient was prepared in 35ml sealable ultracentrifuge tubes (Sorvall Instruments, Dupont, DE) using 5ml of six sucrose solutions of densities 1.09, 1.13, 1.16, 1.20, 1.24 and 1.28g cm\(^{-3}\) (Table 2.2.). All the sucrose solutions contained 1mM EDTA, 0.1% (v/v) ethanol and 5U ml\(^{-1}\) heparin. 5ml of the lowest density solution were placed in the ultracentrifuge tube via a fine plastic tube attached to a 10ml syringe. Then, progressing from the least to the most dense sucrose solution, 5ml of each were introduced carefully under the previous one. Finally 2ml of the cushion solution (1.32g cm\(^{-3}\)) were added. 3ml of the PNS were layered on to the top of the gradient. Any remaining PNS was stored at -20°C.

The discontinuous gradient was centrifuged in a Sorvall TV850 rotor (Dupont, DE) at 45000g for 45 minutes at 4°C to form a continuous gradient. After centrifugation, the bottom of the ultracentrifuge tube was pierced and the gradient was collected as fifteen fractions of approximately equal volume into preweighed tubes on ice. These tubes were weighed to determine the weight of each fraction and the refractive index of each sample was measured using a light refractometer (Atago Co. Ltd., Japan). The density of each fraction was determined from its weight.
Table 2.2. Solutions for the Linear Sucrose Density Gradient.

<table>
<thead>
<tr>
<th>Sucrose (g)</th>
<th>Distilled water (ml)(^a)</th>
<th>Density (g cm(^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>20.6</td>
<td>92.0</td>
<td>1.09</td>
</tr>
<tr>
<td>28.8</td>
<td>88.7</td>
<td>1.13</td>
</tr>
<tr>
<td>36.4</td>
<td>86.2</td>
<td>1.16</td>
</tr>
<tr>
<td>43.6</td>
<td>83.3</td>
<td>1.20</td>
</tr>
<tr>
<td>50.7</td>
<td>80.5</td>
<td>1.24</td>
</tr>
<tr>
<td>57.3</td>
<td>78.1</td>
<td>1.28</td>
</tr>
<tr>
<td>64.1</td>
<td>75.6</td>
<td>1.32</td>
</tr>
</tbody>
</table>

\(^a\) The volume of water required to make a sucrose solution with mass 100g.
and refractive index using a computer programme available in the Microbiology and Immunology Department at Leicester University. The fractions were stored at -20°C.

2.15.3. Enzyme Assays.

Marker enzymes were assayed to determine the distribution of subcellular organelles and lysosomes within the gradient. All assays were carried out in duplicate on each fraction and, in addition, the assays were performed in duplicate with the PNS. Control samples consisted of SVEH and were included in every enzyme assay.

a. Hydrolases.

The lysosomal hydrolases, β-glucuronidase, β-galactosidase and N-acetyl-β-glucosaminidase, and the marker for the endoplasmic reticulum, neutral-α-glucosidase, were assayed fluorimetrically (Peters et al., 1972). The assay was based on the formation of the fluorescent compound 4-methyl-umbelliferone in the presence of the hydrolases.

1. β-glucuronidase.

Substrate: 10mM 4-methyl-umbelliferyl β-glucuronate in dry methoxyethanol.
Assay Buffer: 0.1M sodium acetate, pH4.3 + 0.1% (v/v) Triton X-100.

2. β-galactosidase.

Substrate: 10mM 4-methyl-umbelliferyl β-galactoside in dry methoxyethanol.
Assay Buffer: 0.1M sodium acetate, pH4.3 + 0.1% (v/v) Triton X-100.


Substrate: 10mM 4-methyl-umbelliferyl N-acetyl-β-glucosamine in dry methoxyethanol.
Assay Buffer: 0.1M sodium acetate, pH4.3 + 0.1% (v/v) Triton X-100.


Substrate: 10mM 4-methyl-umbelliferyl neutral-α-glucoside in dry methoxyethanol.
Assay Buffer: 0.1M cacodylate, pH7.5 + 0.1% (v/v) Triton X-100.

The stock substrates were kept at -20°C for up to one month. 200μl of the stock substrate were mixed with 9.8μl of the corresponding buffer to give a working substrate. The working substrate was warmed to 37°C and the assay was started by adding 250μl substrate to 100μl of
the test sample. The mixture was incubated at 37°C for 10 minutes and then the reaction was stopped by the addition of 2ml ice-cold 10mM glycine-NaOH, pH10.4. The assay mixture was left at room temperature for 5 minutes before the fluorescence was determined with an LS-2B Filter Fluorimeter (Perkin Elmer, U.K.) at an excitation wavelength of 365nm and an analysis wavelength of 460nm.

The fluorescent compound, quinine sulphate, in 0.05M sulphuric acid, was used to standardise the assays. 2.5µg ml⁻¹ quinine sulphate gave the same fluorescence value as 0.1nmol 4-methyl-umbelliferone ml⁻¹. This value was used to standardise the assay, with fluorescence values being converted to activity values defined as nmol 4-methyl-umbelliferone formed ml⁻¹ min⁻¹.

b. Lysozyme.

Lysozyme was assayed turbidimetrically using a modification of the method described by Parry et al (1965). The assay was based on lysozyme lysing a suspension of killed *Micrococcus lysodeikticus*, which caused a decrease in turbidity and was measured as absorbance.

The assay was performed in a plastic microtitre plate with flat bottomed wells. The substrate was 16mg *M. lysodeikticus* (U.V. killed) in 10ml 0.067M sodium phosphate buffer, pH6.2. The substrate was sonicated for three 5 second bursts at 40W (Ultrasonic Engineering, U.K.) and was then left at room temperature for 4 hours. The assay reaction was started by adding 200µl of test sample to 100µl of the substrate. This mixture was incubated at 37°C for 10 minutes and the reaction was stopped by the addition of 200µl ice-cold assay buffer. The samples were read immediately using an MR600 Microplate Reader (Dynatech, U.K.) at an analysis wavelength of 450nm and a reference wavelength of 510nm against an air blank. The absorbance readings from the samples were subtracted from those of the control samples to give relative enzyme activity.

c. Catalase.

Catalase, the marker enzyme for peroxisomes, was assayed using the modified method of Peters et al (1972). The assay was based on the reduction of titanium (IV) sulphate to titanium
(II) sulphate by hydrogen peroxide. In the absence of catalase, titanium (II) sulphate was formed as a yellow salt. In the presence of catalase, hydrogen peroxide was broken down and titanium (IV) sulphate remained as a colourless salt. The change in colour was measured as absorbance.

The assay was performed in a plastic microtitre plate with flat bottomed wells. The substrate consisted of 50mg bovine serum albumin, 5ml 0.2M imidazole-HCl, pH7.0, 1.25ml 10% (v/v) Triton X-100 and 750µl 30% (v/v) hydrogen peroxide in a total volume of 50ml distilled water. The reaction was started by mixing 15µl of the test sample with 30µl of the substrate, the samples were then incubated for 10 minutes at room temperature. The reaction was stopped by the addition of 200µl 15% (w/w) titanium (IV) sulphate in 1M sulphuric acid. The absorbance was read using an MR600 Microplate Reader at an analysis wavelength of 410nm and a reference wavelength of 450nm against an air blank.

d. 5' Nucleotidase.

The activity of 5' nucleotidase was used to detect the plasma membrane. The assay was a modification of the procedure described by Segal and Peters (1977). It was based on the release of radiolabelled adenosine, from radiolabelled adenosine 5' monophosphate, by the enzyme.

6.4mM radiolabelled adenosine 5' monophosphate ([β-^14C]AMP, 55mCi mmol^-1, Amersham, U.K.) was diluted 1 in 16 in 50% (v/v) ethanol for use as the substrate. The substrate was diluted into 60mM piperazine-HCl buffer, pH9.0, 24mM magnesium chloride, 12mM β-glycerophosphate, 0.1% (v/v) Triton X-100 and 0.12mM nonradioactive AMP to give a final concentration of 0.1mM [^14C] AMP and a final volume of 50ml.

The assay was started with the addition of 500µl substrate to 100µl test sample. After a 60 minute incubation at 37°C, 250µl 0.2M zinc sulphate were mixed with the samples on ice. 190µl 0.25M barium hydroxide were then added which precipitated any remaining labelled adenosine 5' monophosphate. (The amount of barium hydroxide that was added to the samples was determined by titrating 250µl zinc sulphate against a volume of barium hydroxide until the pH reached 7.0.) The samples were incubated for 30 minutes on ice and were then centrifuged at 1500g for 10 minutes to sediment the precipitated adenosine 5' monophosphate. The supernatants (800µl) were added to 4ml Optiphase Safe scintillation fluid (Pharwall, U.K.) and the
radioactivity was counted for three 5 minute periods in a scintillation counter (Minaxi Tri-carb 4000 series, United Technologies, Packard, U.K.). 800μl of a standard solution, consisting of 500μl substrate plus 540μl distilled water, were also counted. The counts per minute gave the relative activity of the enzyme in each test sample.

e. Malate dehydrogenase.

The marker enzyme for the mitochondria was assayed as described by Kane and Peters (1975), with some modifications. The assay was based on the formation of NAD⁺, from oxaloacetate and NADH, by the enzyme and its measurement as a fluorescent product.

The substrate consisted of 3.7mg oxaloacetate in 10ml 0.1M sodium phosphate buffer, pH7.4, containing 18mg dithiothreitol and 0.01% (v/v) Triton X-100. 500μl of the substrate mix were added to 0.5mg NADH, 5mg bovine serum albumin and 4.5ml phosphate buffer. The assay was started by adding 250μl substrate mixture to 100μl test sample. The samples were incubated at 37°C for 10 minutes and then the reaction was stopped by the addition of 50μl 1.2M hydrochloric acid followed by 200μl 11M sodium hydroxide. The samples were left in the dark at room temperature for 60 minutes. 2.5ml distilled water were then added to each sample and the fluorescence measured at an excitation wavelength of 365nm and an analysis wavelength of 460nm using an LS-2B Filter Fluorimeter. The fluorescence values gave the relative activity of the enzyme in each sample.

f. Fluorescent Protein Assay.

The amount of protein in each test sample was estimated using a fluorescent protein assay, as described previously by Peters et al (1972).

A 1mM stock solution of eosin was prepared in 250ml 50% (v/v) ethanol in distilled water. Before use, 750μl stock solution were diluted in 50ml 0.1M citrate buffer, pH3.0, and were stored on ice. The assay was started by mixing 100μl test sample with 150μl SVEH and 2ml working substrate. After incubation for 10 minutes at room temperature the fluorescence of the samples was measured at an excitation wavelength of 519nm and an analysis wavelength of 540nm using an LS-2B Filter Fluorimeter.
A calibration curve was constructed using bovine serum albumin dissolved in SVEH as the protein source. Concentrations used were in the range of 0 to 200μg ml⁻¹. From the standard curve the fluorescence values of the test samples were converted into units of μg protein ml⁻¹.

2.15.4. Isolation of Lysosome-enriched Fractions.

Results from the analytical isopycnic ultracentrifugation were expressed as frequency density histograms, as described by Peters (1976) using software available in the Microbiology and Immunology Department at Leicester University. For each enzyme, the data were plotted as relative enzyme activity against fraction densities. From these histograms it could be determined which sucrose solutions to use in the preparative discontinuous sucrose density gradient, as the density to which the organelles sedimented could be visualised. Sucrose solutions were chosen so that lysosomes would be separated from other organelles in the PNS.

Macrophages were collected from vaccinated and non-vaccinated guinea pigs and were homogenised (section 2.15.1.). A preparative discontinuous sucrose density gradient was constructed using sucrose solutions of 1.30, 1.18 and 1.13 g cm⁻³, prepared as described above (section 2.15.2.).

<table>
<thead>
<tr>
<th>Sucrose (g)</th>
<th>Distilled water (ml)</th>
<th>Density (g cm⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>61.28</td>
<td>76.9</td>
<td>1.30</td>
</tr>
<tr>
<td>38.76</td>
<td>84.8</td>
<td>1.18</td>
</tr>
<tr>
<td>28.8</td>
<td>88.7</td>
<td>1.13</td>
</tr>
</tbody>
</table>

The gradient contained 10ml of each sucrose solution and 4ml of the PNS and was centrifuged in a Sorvall TV850 rotor (Dupont, DE) at 45000g for 45 minutes at 4°C. Control gradients were prepared with SVEH layered on to the top of the gradient instead of the PNS and were centrifuged under exactly the same conditions. Following centrifugation, the material at the interface of each sucrose solution was collected and the weight and the refractive index of each sample was recorded. The fractions were stored at -20°C.

Enzyme assays were carried out to ascertain whether the lysosomal and non-lysosomal
enzymes appeared on the gradient at the anticipated densities. N-acetyl-β-glucosaminidase, neutral-α-glucosidase, 5' nucleotidase, malate dehydrogenase and lysozyme were assayed as described in section 2.15.3.

2.15.5. Acid Extraction.

The fractions that contained the lysosomes (density 1.18 g cm\(^{-2}\)) and the corresponding control fractions were sonicated at low power (Ultrasonic Engineering, U.K.) for five seconds, whilst on ice, to disrupt the lysosomes. The fractions were then extracted with 5 ml 0.05 M glycine-HCl, pH 2.5, for 40 minutes at room temperature (Gabay et al., 1986). The extracts were ultracentrifuged using a Sorvall AH627 rotor (Dupont, DE) at 10000 g for 20 minutes at 4°C. The supernatants were retained as a source of possible antimycobacterial factors and were stored at -20°C. The protein content of the supernatants was assayed with the fluorescent protein assay (section 2.15.3.).

2.15.6. Antimycobacterial Activity of Lysosomal Extracts.

The assay conditions used were based on those described by Gabay et al. (1986). The assays were performed in 0.1 M citrate buffer, pH 5.5, or 0.05 M phosphate buffer, pH 7.0.

The supernatants from the acid-extracted fractions were diluted in each assay buffer in duplicate to give a final protein concentration of 300 μg ml\(^{-1}\). 250 μl of \(M. \) tuberculosi\(s\) H37Rv, prepared as described in section 2.7. and resuspended in the appropriate buffer, were added to 250 μl of the lysosomal fractions to give a concentration of 2x10\(^5\) bacilli ml\(^{-1}\). As a control, 1x10\(^5\) bacilli were incubated in 500 μl of each assay buffer in duplicate.

The mycobacteria were incubated in each acid-extracted supernatant for 0, 2 and 24 hours at 37°C, after which they were sonicated at 40 W for three periods of 5 seconds to disrupt clumps. A viable count assay was then performed on each fraction (section 2.8.).

2.15.7. The Effect of Heating on Antimycobacterial Activity.

The effect of heating the supernatants was tested by placing aliquots of the acid extracts in a boiling water bath for 15 minutes before the antimycobacterial activity was determined.
2.16. Investigating Reactive Nitrogen Intermediates (RNI) as Mycobactericidal Agents.

2.16.1. Effect of Nitrite on Mycobacterial Viability.

These assays were done in collaboration with J. Carmichael (Department of Microbiology and Immunology, University of Leicester). Some of the results have been reported before (Carmichael, 1992). By combining the results it was possible to test *M. bovis* 81470 and the eight strains of *M. tuberculosis* in triplicate. *Mycobacterium tuberculosis* strain 24, 24 (p16R1) and 24 (pYZ66) were also assayed for their susceptibility to nitrite.

The experiments were performed in reconstituted powdered Eagles minimum essential medium without phenol red, glutamine and sodium bicarbonate (Flow Laboratories, CA). The medium was supplemented with 110 mg l⁻¹ sodium pyruvate, 3.5g l⁻¹ glucose (Fisons, U.K.), 584mg l⁻¹ glutamine, 15mM Hepes and 10% (v/v) HINCS. The pH was adjusted to 5.0 or 7.0. The medium was filter sterilised and stored at 4°C for use within seven days. This final growth medium is referred to as MEM.

Sodium nitrite was diluted from a freshly-made, sterile solution of 5mg ml⁻¹ to give solutions with final concentrations of 2.5mg, 1.25mg, 0.5mg and 0.05mg ml⁻¹ in sterile nanopure water. Mycobacteria were prepared as described (section 2.7.) in MEM, pH5.0 or pH7.0, at a concentration of 1x10⁸ bacilli ml⁻¹.

1x10⁷ bacteria were added to 100µl of each nitrite solution and 800µl MEM at pH5.0 or pH7.0, as appropriate. The controls consisted of 1x10⁷ mycobacteria plus 900µl MEM, pH5.0 or pH7.0. The suspensions were incubated at 37°C and 5% (v/v) CO₂ and viable count assays were performed after 0 and 24 hours (section 2.8.). After the 24 hour incubation period, the mycobacteria were sonicated for three 5 second bursts (Ultrasonic Engineering, U.K.) to ensure single cells were plated.
2.16.2. Addition of Iron to the Nitrite Assay.

The effect of supplementing iron on the survival of mycobacteria, in the presence of 125µg ml⁻¹ sodium nitrite at pH5.0, was assayed.

A fresh, sterile solution of ferric ammonium citrate (5mg ml⁻¹) was diluted in sterile nanopure water to 2.0mg, 1.5mg, 1.0mg, and 0.5mg ml⁻¹. 100µl of each ferric ammonium citrate solution were added to 1x10⁷ mycobacteria, 100µl sodium nitrite at 1.25mg ml⁻¹ sterile nanopure water and 800µl MEM, pH5.0. For controls, 100µl MEM, pH5.0, were included instead of ferric ammonium citrate. The suspensions were then treated exactly as described above (section 2.16.1.).

2.16.3. Addition of Catalase to the Nitrite Assay.

The effect on mycobacterial survival of supplementing catalase, in the presence of 125µg ml⁻¹ sodium nitrite at pH5.0, was tested.

A freshly-made, sterile catalase solution (10000U ml⁻¹) was diluted in sterile nanopure water to 4000U, 2000U and 400U ml⁻¹. 125µl of each catalase solution were added to 1x10⁷ mycobacteria, 100µl sodium nitrite at 1.25mg ml⁻¹ sterile nanopure water and 775µl MEM, pH5.0. 125µl MEM, pH5.0, were added to the controls instead of catalase. The suspensions were subsequently treated as above (section 2.16.1.).

2.16.4. Effect of SIN-1 on Mycobacterial Viability.

The eight strains of M. tuberculosis were tested for susceptibility to SIN-1 (kindly donated by Dr Rainer Henning, Cassella AG, Germany) over 24 hours.

Mycobacteria were prepared as described (section 2.7.) in MEM, pH7.0, at a concentration of 1x10⁸ bacilli ml⁻¹. SIN-1 was diluted from a freshly-made, sterile solution of 50mg ml⁻¹ to give solutions with final concentrations of 25mg, 10mg, 5mg, 2mg and 1mg ml⁻¹ MEM, pH7.0.

1x10⁷ bacteria were added to 100µl of each SIN-1 solution and 800µl MEM, pH7.0. The controls consisted of 1x10⁷ mycobacteria plus 900µl MEM, pH7.0. The suspensions were incubated at 37°C and 5% (v/v) CO₂ and viable count assays were performed after 0 and 24
hours (section 2.8.). After the 24 hour incubation period the mycobacteria were sonicated to ensure single cells were plated.

2.16.5. Addition of Catalase to the SIN-1 Assay.

The effect on mycobacterial survival of supplementing catalase, in the presence of 500µg ml⁻¹ SIN-1, was assayed.

A fresh, sterile catalase solution (10000 U ml⁻¹) was diluted in sterile nanopure water to 4000 U ml⁻¹. 125µl of the catalase solution were added to 1x10⁷ mycobacteria, 100µl of SIN-1 at 5mg ml⁻¹ sterile nanopure water and 775µl MEM, pH7.0. 125µl MEM, pH7.0, were added to the controls instead of catalase. The suspensions were subsequently treated as above (section 2.16.4.).

2.16.6. Addition of Mannitol to the SIN-1 Assay.

The effect on mycobacterial survival of supplementing mannitol, in the presence of 500µg ml⁻¹ SIN-1, was tested.

A freshly-made, sterile mannitol solution (2M) was diluted in 100µl of sterile nanopure water to 1000mM, 500mM, 250mM and 100mM. Each mannitol solution was added to 1x10⁷ mycobacteria, 100µl of SIN-1 at 5mg ml⁻¹ sterile nanopure water and 800µl MEM, pH7.0. 100µl MEM, pH7.0, were added to the controls instead of mannitol. The suspensions were subsequently treated as above (section 2.16.4.).

2.16.7. Measurement of Nitrite Production by Guinea Pig Alveolar Macrophages.

These experiments were an adaptation of those done by Carmichael (1992). The concentration of nitrite (NO₂⁻) in the culture supernatants was determined spectrophotometrically using the Greiss reagent (Green et al, 1982). NO₂⁻ reacts with the Greiss reagent to form a purple azo dye, the absorbance of which is directly proportional to the concentration of NO₂⁻ present in the sample. The Greiss reagent consists of one part 0.1% (w/v) N-naphthylethlenediamine dihydrochloride in distilled water plus one part 1% (w/v)
sulfanilamide in 5% (v/v) orthophosphoric acid. Each part was stored separately at 4°C. The two were mixed together just before use and were kept on ice.

Macrophages were collected using method two (section 2.11.). 1x10⁶ cells were plated out in 24 well tissue culture plates in 1ml RPMI 1640 plus HINCS. After incubation at 37°C and 5% (v/v) CO₂ for 1 hour to allow macrophage adherence, non-adherent cells were removed by two gentle washes in 1ml HBSS-Hepes. 1ml of fresh RPMI 1640 plus HINCS was then added to the monolayers and the plates were reincubated at 37°C and 5% (v/v) CO₂.

250µl of the culture supernatants were collected at the appropriate time points (24 or 48 hours), mixed with 1ml of Greiss reagent and incubated for 10 minutes at room temperature. The absorbance was measured at 546nm using distilled water as the blank (PU8720 UV/VIS Scanning Spectrophotometer, Philips, U.K.).


Concentrations of sodium nitrite, ranging from 0-100µM, were prepared in duplicate in 250µl distilled water. 1ml of Greiss reagent was added to each standard and the absorbance was measured at 546nm after the 10 minute incubation at room temperature. A calibration curve was constructed by plotting absorbance against the concentration of NO₂⁻.

A NO₂⁻ standard assay was included in every experiment that measured cellular NO₂⁻ production. The absorbance of RPMI 1640 plus HINCS alone was determined in each experiment. The latter was subtracted from the absorbance values of the samples, the concentration of NO₂⁻ in the macrophage culture supernatants was then calculated from the standard curve.

2.16.9. Stimulation of Nitrite Production with Mycobacteria.

Mycobacteria (strains H37Ra, B1453, H37Rv, 79499 and bovis) were harvested and washed, as described in section 2.7. The concentration was adjusted to 2.5x10⁹ bacilli ml⁻¹ in RPMI 1640 plus HINCS. After the guinea pig macrophages had adhered and had been washed (section 2.16.7.), half of the macrophage monolayers were infected with 25x10⁶ mycobacteria giving a bacterium to cell ratio of 25:1. Control wells, consisting of 25x10⁶ mycobacteria in 1ml RPMI 1640 plus HINCS, were included to monitor any NO₂⁻ production by the bacteria. The 24
well plates were incubated at 37°C and 5% (v/v) CO₂ for the appropriate length of time and at each time point the supernatant was removed and its NO²⁻ concentration assayed, as described (section 2.16.7.). The supernatants from wells that had contained mycobacteria were filtered through 0.22µm filters (Millipore, MA) before testing, to ensure the removal of virulent bacteria.

2.16.10. Stimulation of Nitrite Production with LPS.

Guinea pig macrophages were allowed to adhere, were washed in HBSS-Hepes and then 1ml RPMI 1640 plus HINCS was added to each well (section 2.16.7.). A fresh, sterile solution of *Escherichia coli* LPS (1µg ml⁻¹ RPMI 1640 plus HINCS) was added to half of the monolayers to give a final concentration of 10µg ml⁻¹. Cell-free control wells which consisted of 10 µg ml⁻¹ LPS in 1ml RPMI 1640 plus HINCS were also included. The 24 well plates were incubated at 37°C and 5% (v/v) CO₂ for the appropriate length of time and at each time point the supernatant was removed and tested for NO₂⁻, as described (section 2.16.7.).

2.16.11. Measurement of Nitrite Production by U937.

Two cultures of U937s were grown, as described (section 2.4.1.). One of the cultures was activated with IFN-γ for 48 hours (section 2.4.1.). After this time, both the activated and non-activated cells were harvested and counted (section 2.4.1.). 1x10⁶ cells were dispensed into 24 well plates in a final volume of 1ml RPMI 1640 plus HIFCS. The plates were incubated at 37°C and 5% (v/v) CO₂.

NO₂⁻ production was tested over 48 hours (section 2.16.7.). Mycobacteria, prepared as described in section 2.7. and at a concentration of 25x10⁶ bacilli well⁻¹, were included as potential stimulants of NO₂⁻ production in certain experiments. In other experiments *E. coli* LPS was added (section 2.16.10). The supernatants from wells that had contained mycobacteria were filtered through 0.22µm filters (Millipore, MA) before testing for NO₂⁻.


The macrophages were activated with IFN-γ for 48 hours as described in section 2.4.2.
16×10⁵ mycobacteria (section 2.7.) were then added to certain wells to induce NO₂⁻ production. Alternatively, 10µg of *E. coli* LPS were included. The concentration of NO₂⁻ was determined after a further 24 hours of incubation (section 2.16.7.). Supernatants that contained mycobacteria were filtered before testing (section 2.16.7.).

2.16.13. Inhibition of Nitrite Production by J774s with N⁵-MMA.

The experiment was repeated as described in section 2.16.12. The inhibitor was added to the macrophages after the 48 hour activation period. A 10mM solution of N⁵-monomethyl-L-arginine (N⁵-MMA) was made in 1ml of distilled water and was filter sterilised. 50µl of this solution were included in the medium of the J774 monolayers, giving a final concentration of 500µM well⁻¹.


This experiment was adapted from the procedures described by O'Brien and Andrew (1991) and O'Brien *et al.* (1991).

Alveolar macrophages were collected from vaccinated guinea pigs using method two (section 2.11). 5×10⁵ cells, in 500µl RPMI 1640 plus HINCS, were plated out into 24 well tissue culture plates. After being incubated for 1 hour at 37°C and 5% (v/v) CO₂, the cells were washed once in HBSS-Hepes and 400µl of fresh RPMI 1640 plus HINCS were added to each monolayer.

*Mycobacterium tuberculosis* B1453 was prepared at a concentration of 50×10⁶ bacilli ml⁻¹ in RPMI 1640 plus HINCS (section 2.7.). 50µl of the suspension were used to infect each monolayer, giving a bacteria to cell ratio of five to one. 25×10⁵ mycobacteria were also added to 450µl of medium alone. 50µl of a 10mM solution of N⁵-MMA (section 2.16.13.) were then added to half of the wells to give a final concentration of 500µM. 50µl of RPMI 1640 plus HINCS were added to the remaining wells. The macrophages were reincubated for two hours to allow phagocytosis. After this time each monolayer was washed six times in 1ml HBSS-Hepes to ensure the removal of extracellular mycobacteria. 450µl of RPMI 1640 plus HINCS were added to half of the monolayers and 500µM N⁵-MMA in 50µl of water or 50µl of medium alone were included in
the appropriate wells. These monolayers were then incubated for a further 24 hours at 37°C and 5% (v/v) CO₂.

The supernatants from the remaining wells were reserved. 500μl of a sterile, saturated solution of digitonin (8mg ml⁻¹ HBSS-Hepes) were added to the macrophages and the monolayers were incubated for 10 minutes at 37°C and 5% (v/v) CO₂. This treatment ensured lysis of the cells. Both the supernatants and cell suspensions were then sonicated for three 5 second bursts (Ultrasonic Engineering, U.K.) to disrupt any mycobacteria that had clumped. Finally, viable count assays (section 2.8.) were performed to ascertain the number of intracellular and extracellular mycobacteria associated with each macrophage monolayer. Mycobacteria incubated in medium alone were also subjected to viable count assays (section 2.8.).

Those wells that were incubated for a further 24 hours were treated exactly as described above, except that 500μl of digitonin were added to the reserved supernatants. The supernatants were incubated for 10 minutes at 37°C and 5% (v/v) CO₂. Thus, any cells that had detached during the 24 hour incubation period were lysed and this allowed all the viable mycobacteria to be accounted for.

2.17. Investigating the Enzyme Ornithine Decarboxylase (ODC) as Part of a Mycobactericidal Pathway.

2.17.1. ODC Content of U937s.

Activated and non-activated cultures of U937s were prepared, as described (section 2.4.1.). The cells were harvested, washed once in HBSS-Hepes by re-centrifugation and were counted microscopically (section 2.4.3.). 2x10⁵, 5x10⁵, 8x10⁵, 1x10⁶, 2x10⁶ and 5x10⁶ cells were aliquoted into duplicate sterile tubes. The volume was then made up to 3ml with 18mM Tris-HCl (pH7.2 at 37°C). The cell suspension was freeze-thawed, using dry ice and a 37°C water bath, until the cells were at least 90% disrupted, as determined microscopically (section 2.4.3.). Typically, three cycles of freeze-thawing were required. The homogenised cells were then ultracentrifuged at 30000g for 30 minutes at 4°C using a Sorvall AH627 rotor (Dupont)
and the supernatants assayed for ODC activity as follows.

The method used to measure ODC was an adaptation of that described by Slotkin and Bartolome (1983) and Sharp (1991). 500μl of each supernatant was dispensed in duplicate into glass scintillation vials. 50μl of freshly-made 40M dithiothreitol and 50μl of freshly-made 1M pyridoxal 5’ phosphate were added to the supernatants. The dithiothreitol was always added before the pyridoxal 5’ phosphate. 100μl of 10% (v/v) methylbenzethonium hydroxide in methanol were pipetted into 500μl eppendorf tubes that contained rolled up Whatman No.1 filter paper (Whatman International Ltd., U.K.) of specific dimensions (Fig. 2.1.). The dimensions of the filter paper were found to be crucial for a reproducible assay. Care was taken to ensure all the methylbenzethonium hydroxide was absorbed onto the filter paper. The 500μl eppendorf tubes were placed inside 1500μl eppendorf tubes and these were in turn placed into the scintillation vials. For easier manipulation both the 500μl and 1500μl eppendorf tubes had previously had their lids cut off. Serum stoppers were used to seal the vials and they were then incubated at 37°C for 5 minutes.

The reaction was started by injecting 5nmol of L-[carboxyl-14C] ornithine (50mCi mmole⁻¹, Amersham, U.K.) in a total volume of 400μl of distilled water through the serum stopper into the incubation mixture. The vials were then incubated at 37°C for 60 minutes. The reaction was stopped by injection of 500μl of 10% (w/v) trichloroacetic acid into the incubation mixture. The vials were placed on ice for 3 minutes and were then incubated at 37°C for another 30 minutes to ensure all the liberated 14CO₂ was absorbed onto the filter paper. After this, the filter papers were removed from the eppendorf tubes and were placed into scintillation vial inserts. They were left to dry overnight at room temperature. 4ml of Optiphase Safe scintillation fluid (Pharwall, U.K.) were added to each insert and the 14C was counted three times over a period of 5 minutes in a scintillation counter (Minaxi Tri-carb 4000 series, United Technologies, Packard, U.K.).

2.17.2. ODC Standard Curve.

*Escherichia coli* ODC (1U mg⁻¹ protein. One unit releases 1μmole CO₂ from L-ornithine min⁻¹ at pH5.2 and 37°C) was reconstituted in 18mM Tris-HCl (pH7.2 at 37°C) to give a final
Fig. 2.1. The Dimensions of the Whatman No.1 Filter Paper for Use in the ODC Assay.
concentration of 1U ml\(^{-1}\). This stock solution was stored at -20°C. For the standard curve, amounts of ODC, ranging from 0-50mU, were prepared in duplicate in 500\(\mu\)l 18mM Tris-HCI (pH7.2 at 37°C). ODC activity was then measured as described above (section 2.17.1.). In addition, 100\(\mu\)l of the incubation mixture from the vial containing no ODC was pipetted onto Whatman No.1 filter paper after the assay had finished. This allowed the total 14C count to be determined.

An ODC standard curve was included in every assay. A calibration curve was then constructed by plotting counts per minute against ODC concentration.

2.17.3. ODC Content of Alveolar Macrophages.

Macrophages from vaccinated and non-vaccinated guinea pigs were collected using method 1 (section 2.11.). 2x10^6 cells, from each guinea pig, were aliquoted into sterile universal tubes and the volume made up to 3ml with 18mM Tris-HCI (pH7.2 at 37°C). The cell suspension was freeze-thawed and ultracentrifuged, as described in section 2.17.1., and the supernatant was then assayed for ODC content, again as described (section 2.17.1.).

Alternatively, vaccinated guinea pigs were killed 24 hours after the intravenous injection of BCG (section 2.10.) and the ODC content of 2x10^6 alveolar macrophages assayed then.

2.17.4. ODC Content of BCG-stimulated Alveolar Macrophages.

Macrophages from vaccinated guinea pigs were collected using method 2 (section 2.11.). 2x10^6 cells were plated out into 24 well plates in a total volume of 1ml RPMI 1640 plus HINCS. The macrophages were allowed to adhere for 1 hour at 37°C and 5% (v/v) CO\(_2\). Non-adherent cells were removed by two washes in medium and then 1ml RPMI 1640 plus HINCS was added to each well. 5x10^7 \(M.\ bovis\) BCG (prepared as described in section 2.7.) were included in the medium of half of the wells. The macrophages were then incubated for 24 hours at 37°C and 5% (v/v) CO\(_2\).

After this time, the medium was removed and the monolayers were washed six times in HBSS-Hepes. The integrity of the monolayer was assessed microscopically before the cells were scraped from the surface of the well with a plunger from a 1ml syringe. Microscopic observation
ensured all the macrophages had been removed from the surface. The cells were then aliquoted into sterile universal tubes and the volume made up to 3ml with 18mM Tris-HCl (pH7.2 at 37°C). After freeze-thawing and ultracentrifugation, the ODC content of the macrophages was ascertained (section 2.17.1.).

2.18. Investigating the Susceptibility of Mouse-passaged \( \textit{M. tuberculosis} \) H37Rv to Toxic Agents.

The viability of pre-passaged and post-passaged H37Rv (section 2.5.), in the presence of hydrogen peroxide and in the presence of nitrite, was tested.

2.18.1. Susceptibility to Hydrogen Peroxide.

These assays were based on those described by Jackett et al (1981). The experiments were performed in citrate-phosphate buffer (1:1 mix of 50mM citric acid and 50mM sodium phosphate). The pH of 10ml aliquots of the buffer was adjusted to 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 or 4.0. The H37Rv (pre-passaged or post-passaged, as appropriate) was prepared, as described in section 2.7., at a concentration of \( 2 \times 10^7 \, \text{ml}^{-1} \) in nanopure water. \( 1 \times 10^6 \) mycobacteria were added to 950\( \mu \)l citrate-phosphate buffer at the above pH values. Hydrogen peroxide was then added to the suspensions to give a final concentration of 0.08% (v/v). Controls contained no hydrogen peroxide. The suspensions were incubated at 37°C for 90 minutes. Viable count assays (section 2.8.) were then performed.

2.18.2. Susceptibility to Nitrite.

The effect of nitrite on the viability of the pre- and post-passaged strains was tested exactly as described in section 2.16.1.

2.19. Demonstrating an Acid Tolerance Response (ATR) with \( \textit{M. smegmatis} \).

These methods were based on those described by Foster and Hall (1990). As the effect of pH stress was being tested, non-sonicated mycobacteria were used in the experiments because
sonication would have been an additional stress on the bacteria.


*Mycobacterium smegmatis* was grown overnight in 50ml 7H9 plus ADC, pH7.6, at 37°C and with shaking. 1ml of the culture was sonicated (Ultrasonic Engineering, U.K.) for three 5 second bursts and counted microscopically with a Thoma chamber. Two 50ml volumes of 7H9 plus ADC, pH7.6, were inoculated with 5x10⁷ non-sonicated mycobacteria from the overnight cultures. The cultures were replaced in the shaking incubator and 1ml samples were taken every hour for 12 hours. At each time point the samples were sonicated and viable count assays (section 2.8.) were performed. From this growth curve the time taken for *M. smegmatis* to reach a concentration of 1x10⁸ bacteria ml⁻¹, at pH7.6, was ascertained. It was found to be 14 hours. The doubling time was 2 hours.

The growth curve at pH7.6 was repeated, as above. When the mycobacterial concentration reached 1x10⁸ ml⁻¹, the pH of the cultures was reduced to 5.8. 25% (v/v) sterile hydrochloric acid was used to decrease the pH. The acid was added slowly, in drops, while the cultures were being swirled by hand, to minimise local acid stress. The cultures were reincubated and 1ml samples were taken every hour for 10 hours and were treated as described above. From this growth curve, the time taken for *M. smegmatis* to double, once the pH had been dropped to 5.8, was found to be 4 hours.

2.19.2. Testing *M. smegmatis* for an ATR.

*Mycobacterium smegmatis* was grown overnight and 5x10⁷ non-sonicated mycobacteria were inoculated into four 50ml volumes of 7H9 plus ADC, pH7.6, as described (section 2.19.1.). The cultures were incubated at 37°C with shaking for 14 hours and then the pH of two was dropped to an adaptation pH of 5.8 (section 2.19.1.) (Foster and Hall, 1990). The cultures at pH7.6 were incubated for a further 2 hours, those at pH5.8 were incubated for 4 hours. These times allowed the mycobacteria to double. Once the bacterial concentration had reached 2x10⁸ ml⁻¹, the pH of the cultures was dropped to a lethal pH of 3.3 with 25% (v/v) sterile hydrochloric acid (Foster and Hall, 1990). The cultures were reincubated and 1ml samples were
taken at 0, 1, 2, 3 and 4 hours. The samples were sonicated and viability tests were then undertaken (section 2.8.).

2.19.3. Survival of *M. smegmatis* over a Range of pH.

It was found that using an adaptation pH of 5.8 and a lethal pH of 3.3 (section 2.19.2.) failed to demonstrate an ATR. The viability of *M. smegmatis*, in 7H9 plus ADC, was therefore tested at different pHs to determine the best pH to use as the adaptation and lethal pH with mycobacteria. Both hydrochloric and phosphoric acid were used to pH the media.

*Mycobacterium smegmatis* mc²155 was grown overnight in 50ml 7H9 plus ADC, pH7.6, at 37°C with shaking. 1ml of the culture was then sonicated and counted. 1x10⁶ non-sonicated mycobacteria were added to 1ml 7H9 plus ADC in duplicate, at the following pHs: 6.0, 5.0, 4.5, 4.0, 3.5, 3.0, 2.5 or 2.0. 25% (v/v) hydrochloric acid was used to pH one set of media and 25% (v/v) phosphoric acid was used to pH another set of media. The cultures were reincubated and, at 0, 2 and 4 hours, the cultures were sonicated and tested for viability (section 2.8.).

From these results, it was decided to use an adaptation pH of 5.0 and a lethal pH of 3.0 with hydrochloric acid and an adaptation pH of 5.0 and a lethal pH of 2.5 with phosphoric acid. pH5.0 was the lowest pH at which the mycobacteria still multiplied. pH3.0 and pH2.5 were lethal to the bacteria over 4 hours.

2.19.4. Testing *M. smegmatis* for an ATR with Hydrochloric Acid.

The experiment was repeated exactly as described in section 2.19.2., except the adaptation pH was 5.0 and the lethal pH was 3.0.

2.19.5. Testing *M. smegmatis* for an ATR with Phosphoric Acid.

The experiment was repeated as described in section 2.19.2., except 25% (v/v) phosphoric acid was used to pH the media, the adaptation pH was 5.0 and the lethal pH was 2.5. It was found that an ATR was not exhibited when a lethal pH of 2.5 was used. The experiment was subsequently repeated with a lethal pH of 3.5.
2.19.6. Determining the Length of the Adaptation Period Required for an ATR.

The experiment was repeated using 25% (v/v) phosphoric acid to pH the medium, an adaptation pH of 5.0 and a lethal pH of 3.5 (section 2.19.5.). In addition, the cultures were left at the adaptation pH for 15, 60 or 120 minutes rather than 4 hours. Non-adapted cultures were incubated for 15, 60 or 120 minutes, once the bacterial concentration had reached $1 \times 10^8$ ml$^{-1}$, before the pH was decreased to 3.5. The experiment was then continued, as described in section 2.19.2.

2.19.7. Testing the Effect of Chloramphenicol on the ATR.

$1 \times 10^9$ non-sonicated M. smegmatis were inoculated from an overnight culture into a number of universals each containing 10ml 7H9 plus ADC, pH5.0. 25% (v/v) phosphoric acid was used to pH the media. Increasing concentrations of chloramphenicol, ranging from 0-100μg ml$^{-1}$, were added to each universal from a stock solution of 5mg ml$^{-1}$ ethanol. The cultures were incubated at 37°C and with shaking for 16 hours before viable count assays were performed (section 2.8.).

It was found that the MIC of chloramphenicol for M. smegmatis (at a concentration of $1 \times 10^8$ bacteria ml$^{-1}$ and at pH5.0) was 20μg ml$^{-1}$. The experiment described in section 2.19.5. was then repeated. 30 minutes before the pH was reduced to 5.0, 200μg ml$^{-1}$ chloramphenicol (stock solution 20mg ml$^{-1}$ ethanol) were added to two cultures and an identical volume of ethanol was added to two control cultures. The experiment was then continued as normal (section 2.19.2.).

2.20. Statistical Analysis.

Data were analysed for significance with the Student's t-test.
3. RESULTS AND DISCUSSION.
3.1. Attempts to Activate Human Monocytes and Macrophages for Antimycobacterial Activity in vitro.

To understand how activated human macrophages kill *M. tuberculosis* in vivo, it is helpful to study this cell in vitro. Ideally, it would be possible to activate the macrophage in vitro to kill *M. tuberculosis*, thereby allowing the tuberculocidal mechanisms to be investigated. However, despite numerous attempts, most researchers have found it impossible to suitably activate human macrophages in vitro (for example see Douvas et al, 1985; Rook et al, 1986a; Steele et al, 1986; Rook, 1990), although some progress has been made with murine macrophages (Rook et al, 1986a; Flesch and Kaufmann, 1987; Chan et al, 1992).

Two groups have published procedures for the in vitro activation of antimycobacterial mechanisms in human monocytes and macrophages (Crowle and May, 1981; Denis, 1991b). Crowle and May (1981) used supernatants from stimulated lymphocytes to activate homologous human macrophages to inhibit the replication of *M. bovis* BCG and *M. tuberculosis* Erdman. Denis (1991b) was able to induce human monocytes to kill *M. tuberculosis* H37Rv by including interferon-γ, tumour necrosis factor-α and the active form of vitamin D₃ (calcitriol) in the culture medium. These are extremely important experiments that may allow the tuberculocidal mechanisms of human macrophages to be elucidated, with implications for disease prevention and therapy. As the main purpose of this thesis was to study the tuberculocidal properties of macrophages, attempts were made to repeat the experiments of Crowle and May (1981) and Denis (1991b).

3.1.1. The Antimycobacterial Activity of Human Monocytes.

It was decided to undertake the experiment of Denis (1991b) first as he had achieved killing of *M. tuberculosis*. He had also employed defined immunomodulators to activate the monocytes rather than an uncharacterised lymphocyte culture supernatant. In addition, his experiment was shorter in length and technically less difficult than that of Crowle and May (1981). The experimental procedure was kept as close as possible to that of Denis (1991b) in order to maximise the chances of obtaining comparable results. Blood from two donors (A. and B.) were used in separate experiments. Both donors had received a BCG vaccination when younger.
Monocyte monolayers were incubated overnight in the presence or absence of 1000U ml\(^{-1}\) interferon-\(\gamma\), 100U ml\(^{-1}\) tumour necrosis factor-\(\alpha\) and 10\(^{-9}\)M calcitriol. \textit{Mycobacterium tuberculosis} H37Rv was then added at an infection ratio of ten bacteria to one monocyte and phagocytosis was allowed to proceed for six hours. After this time extracellular mycobacteria were washed away and the growth of the remaining mycobacteria was monitored for eight days. Thus, immunomodulators were only present overnight and during the infection period.

The results are shown in Tables 3.1. and 3.2. The number of viable bacteria was assessed by counting colony forming units on agar (Table 3.1.). Both the numbers of cell-associated mycobacteria and mycobacteria in the monolayer supernatant were recorded. A complete account of viable \textit{M. tuberculosis} was kept as macrophages that contain more than about five bacilli tend to die (Rook, 1990). Dead monocytes float off from the surface of the well and if the mycobacteria that are inside these cells are not accounted for, then a false killing effect may be observed. In addition, the numbers of intracellular acid-fast bacteria inside one hundred infected monocytes were recorded (Table 3.2.).

It was found that the immunomodulators were not able to activate the monocytes of either donor to kill \textit{M. tuberculosis} H37Rv over an eight day period (Tables 3.1. and 3.2.). The number of viable mycobacteria associated with the monolayer and in the supernatant actually increased with time (Table 3.1. and Fig. 3.1.). Over eight days, there was a 0.25 log increase in the total number of bacteria in the treated monolayers of donor A. Similarly, there was a 0.25 log increase in the total number of bacteria in the untreated monolayers of donor A (Table 3.1.a.). With donor B., there was a 0.15 log increase in the total number of bacteria in the treated monolayers and a 0.19 log increase in the total number of bacteria in the untreated monolayers by day eight (Table 3.1.b.). Thus, \textit{M. tuberculosis} H37Rv was able to multiply to the same extent in treated or untreated monolayers. The immunomodulators therefore failed to induce any antimycobacterial activity in human monocytes. These results are in contrast to those of Denis (1991b). He reported a two-log reduction in the total number of mycobacteria in the wells of treated monolayers at day eight and was therefore able to conclude that the immunomodulators activated human monocytes to kill \textit{M. tuberculosis}.

Even though each monolayer was subjected to six washes with RPMI 1640 medium after
Table 3.1.a. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Colony Counting (Donor A.).

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>MEAN log_{10} CFU ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TREATED(^a)</td>
</tr>
<tr>
<td>0</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>6.80 ± 0.10</td>
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<tr>
<td></td>
<td>Monolayer-associate</td>
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<tr>
<td></td>
<td>6.72 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>Monolayer-supernatant</td>
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<tr>
<td></td>
<td>6.03 ± 0.17</td>
</tr>
<tr>
<td>4</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>6.91 ± 0.07</td>
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<tr>
<td></td>
<td>Monolayer-associate</td>
</tr>
<tr>
<td></td>
<td>6.83 ± 0.12</td>
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<tr>
<td></td>
<td>Monolayer-supernatant</td>
</tr>
<tr>
<td></td>
<td>6.14 ± 0.12</td>
</tr>
<tr>
<td>8</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td>7.05 ± 0.13</td>
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<tr>
<td></td>
<td>Monolayer-associate</td>
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<tr>
<td></td>
<td>6.91 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>Monolayer-supernatant</td>
</tr>
<tr>
<td></td>
<td>6.49 ± 0.18</td>
</tr>
</tbody>
</table>

The results show the total number of bacterial colony forming units (CFU) in the well, the number of monolayer-associated bacteria and the number of bacilli recovered from the monolayer supernatant. All values represent the mean ± S.D. of triplicate wells.

\(^a\)Monolayers were incubated with 1000U ml\(^{-1}\) interferon-γ, 100U ml\(^{-1}\) tumour necrosis factor-α and 10\(^{-6}\)M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
Table 3.1.b. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Colony Counting
(Donor B.).

<table>
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<th>TIME (DAYS)</th>
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<th>TREATED(^a)</th>
<th>UNTREATED</th>
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<td></td>
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<td>MEAN log(_{10}) CFU ± S.D.</td>
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<tr>
<td>0</td>
<td>Total</td>
<td>6.25 ± 0.07</td>
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<tr>
<td></td>
<td>Monolayer associated</td>
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<tr>
<td></td>
<td>Monolayer supernatant</td>
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<tr>
<td>4</td>
<td>Total</td>
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<td></td>
<td>Monolayer associated</td>
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<td></td>
<td>Monolayer supernatant</td>
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<td>Total</td>
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<td>6.43 ± 0.02</td>
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<td>Monolayer associated</td>
<td>6.37 ± 0.05</td>
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<td>Monolayer supernatant</td>
<td>5.33 ± 0.03</td>
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The results show the total number of bacterial colony forming units (CFU) in the well, the number of monolayer-associated bacteria and the number of bacilli recovered from the monolayer supernatant. All values represent the mean ± S.D. of triplicate wells.

\(^a\) Monolayers were incubated with 1000U ml\(^{-1}\) interferon-γ, 100U ml\(^{-1}\) tumour necrosis factor-α and 10\(^{-9}\)M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
Fig. 3.1. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Colony Counting.

Donor A.

![Graph A](image1.png)

Donor B.

![Graph B](image2.png)

The figures show the total number of bacterial colony forming units (CFU) in the well (■), the number of monolayer-associated bacteria (▲) and the number of bacilli recovered from the supernatant (●). Each point is the mean of triplicate wells.

Monocytes were untreated or were incubated with 1000U ml⁻¹ interferon-γ, 100U ml⁻¹ tumour necrosis factor-α and 10⁻⁹M calcitriol before and during infection with H37Rv.
Table 3.2.a. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Acid-fast Staining (Donor A.).

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<th>UNTREATED</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>1.49 ± 0.07</td>
<td>1.55 ± 0.04</td>
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<tr>
<td>4</td>
<td>1.63 ± 0.13</td>
<td>1.77 ± 0.15</td>
</tr>
<tr>
<td>8</td>
<td>2.51 ± 0.07</td>
<td>2.51 ± 0.18</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

\(^a\)Monolayers were incubated with 1000U ml\(^{-1}\) interferon-\(\gamma\), 100U ml\(^{-1}\) tumour necrosis factor-\(\alpha\) and 10\(^{-9}\)M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
Table 3.2.b. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Acid-fast Staining (Donor B.).

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>TREATED&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UNTREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.25 ± 0.04</td>
<td>1.37 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>1.36 ± 0.04</td>
<td>1.56 ± 0.06</td>
</tr>
<tr>
<td>8</td>
<td>2.08 ± 0.06</td>
<td>2.48 ± 0.04</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

<sup>a</sup>Monolayers were incubated with 1000U ml<sup>-1</sup> interferon-γ, 100U ml<sup>-1</sup> tumour necrosis factor-α and 10<sup>-9</sup>M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
Fig. 3.2. The Fate of *M. tuberculosis* H37Rv in Human Monocytes Measured by Acid-fast Staining.

Donor A.

![Graph showing the fate of M. tuberculosis H37Rv in human monocytes measured by acid-fast staining for Donor A.](image)

Donor B.

![Graph showing the fate of M. tuberculosis H37Rv in human monocytes measured by acid-fast staining for Donor B.](image)

Each point is the mean of triplicate wells.

Monocytes were untreated (■) or were incubated with 1000U ml⁻¹ interferon-γ, 100U ml⁻¹ tumour necrosis factor-α and 10⁻²⁹M calcitriol (●) before and during infection with H37Rv.
the phagocytosis period, it appears that a large number of bacteria remained in the supernatant at
time zero (Table 3.1.). However, the extracellular mycobacteria represent approximately 10%
or less of the original infectious inoculum which indicates that the washings were effective in
removing the majority of non-phagocytosed bacilli. *Mycobacterium tuberculosis* is not able to
multiply significantly in the RPMI 1640 medium that was used in these experiments (Crowle,
1988). It must therefore be assumed that the increase in the numbers of bacteria in the
supernatant over the eight day incubation period (Table 3.1.) was predominantly due to the loss of
heavily infected monocytes from the monolayer.

There was no evidence for killing of *M. tuberculosis* H37Rv when the monolayers were
examined visually using the acid-fast stain (Table 3.2. and Fig. 3.2.). With this stain,
intracellular mycobacteria appear red against the pale blue background of the cell. The acid-fast
stain therefore allowed the enumeration of mycobacteria within each infected monocyte. It was
found that the numbers of intracellular mycobacteria increased with time (Table 3.2. and Fig.
3.2.). For example, in the monocytes of donor A., the number of intracellular bacilli increased
over the eight day period by 68% for treated monolayers and 62% for untreated monolayers
(Table 3.2.a.). In the monocytes of donor B., the number of intracellular bacteria had increased
by 66% in treated monolayers and by 81% in untreated monolayers at day eight (Table 3.2.b.).

Staining gives a crude indication of the fate of mycobacteria within cells as it does not
distinguish between viable and non-viable bacilli. Inaccurate results may be obtained because
dead cells detach from the surface. In addition, viable tubercle bacilli can exist as non-acid-fast
forms within macrophages (Crowle, 1988) leading to false conclusions. Dead bacteria can be
visualised if they begin to disintegrate. However, in these experiments disintegrating
mycobacteria were not observed inside any cells. Thus, it can be concluded that
immunomodulator-treated monocytes did not possess increased antimycobacterial activity
compared to untreated monocytes, when assessed by the acid-fast stain.

In a separate experiment, the immunomodulators were tested for their ability to activate
the monocytes for hydrogen peroxide release. Treating the monocytes for twenty-four hours with
the immunomodulators did not activate the cells for significantly increased hydrogen peroxide
production compared to the untreated cells (Table 3.3.). In fact, the treated monocytes showed, on
Table 3.3. Hydrogen Peroxide Production by Human Monocytes.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Treated(^a)</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.25 ± 0.22</td>
<td>1.26 ± 0.06</td>
</tr>
<tr>
<td>PMA</td>
<td>3.97 ± 0.16</td>
<td>4.75 ± 0.02</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>1.48 ± 0.10</td>
<td>1.52 ± 0.00</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of three monolayers from one donor.

\(^a\)Monolayers were incubated overnight with 1000U ml\(^{-1}\) interferon-\(\gamma\), 100U ml\(^{-1}\) tumour necrosis factor-\(\alpha\) and 10\(^{-9}\)M calcitriol.
average, less hydrogen peroxide release than the untreated monocytes. For example, when PMA was used as the stimulus, significantly (P< 0.01) less hydrogen peroxide was produced by immunomodulator-treated cells than untreated cells.

It therefore appears that, in my hands, the combination of interferon-γ, tumour necrosis factor-α and calcitriol does not induce human monocytes to kill *M. tuberculosis* or to release an increased amount of hydrogen peroxide. Hydrogen peroxide production is commonly used as an indication of cellular activation. It seems that, in this case, the three immunomodulators used by Denis were not activating the monocytes.

In a collaborative venture, researchers in London tried to repeat the experiment published by Denis (1991b) (Warwick-Davies et al, 1994). They also found that the immunomodulators did not activate the tuberculocidal activity of human monocytes. However, in the majority of their experiments it was observed that the treated monocytes started to die at day two and that the monolayer was almost completely lysed by day seven. This was in contrast to the experiments performed in Leicester, where approximately 27% of the cells were lost in the treated and untreated monolayers of both donors over the eight day period (Table 3.4.). It was concluded by the group in London that the immunomodulators could sensitise the monocytes to a cytotoxic effect of the tubercle bacilli, leading to the destruction of the monolayer (Warwick-Davies et al, 1994). This may be similar to the ability of virulent *M. tuberculosis* to sensitise human cells to the toxic effect of tumour necrosis factor (Filley and Rook, 1991; Filley et al, 1992). It is not known why the immunomodulator-treated monocytes died in certain experiments but not in others. Contributing factors may have included variations in experimental technique and donor-to-donor variation (section 1.8.1.).

It is not certain why, contrary to the findings of Denis (1991b), the combination of immunomodulators failed to activate the monocytes. It is unlikely that the quality of the immunomodulators was at fault. Interferon-γ was provided in separate batches to each group. Tumour necrosis factor-α and calcitriol were obtained independently from different sources by the two laboratories. It must therefore be concluded that, in my experiments and in those of the London group, some other part of the procedure is failing. Possibly, differences between the donors used in the U.K. and those used by Denis (1991b) in Quebec are responsible for the
Table 3.4.a. The DNA Content of the Monocyte Monolayer (Donor A.).

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>TREATED&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UNTREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.23 ± 0.06</td>
<td>6.08 ± 0.08</td>
</tr>
<tr>
<td>4</td>
<td>5.72 ± 0.14</td>
<td>5.52 ± 0.17</td>
</tr>
<tr>
<td>8</td>
<td>4.30 ± 0.10</td>
<td>4.59 ± 0.15</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

<sup>a</sup>Monolayers were incubated with 1000U ml<sup>-1</sup> interferon-γ, 100U ml<sup>-1</sup> tumour necrosis factor-α and 10<sup>-9</sup>M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
Table 3.4.b. The DNA Content of the Monocyte Monolayer (Donor B.).

<table>
<thead>
<tr>
<th>TIME (DAYS)</th>
<th>TREATED(^a)</th>
<th>UNTREATED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>6.67 ± 0.06</td>
<td>6.06 ± 0.010</td>
</tr>
<tr>
<td>4</td>
<td>5.66 ± 0.15</td>
<td>4.83 ± 0.07</td>
</tr>
<tr>
<td>8</td>
<td>5.14 ± 0.17</td>
<td>4.28 ± 0.22</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

\(^a\)Monolayers were incubated with 1000U ml\(^{-1}\) interferon-γ, 100U ml\(^{-1}\) tumour necrosis factor-α and 10\(^{-9}\)M calcitriol. Immunomodulators were present before and during the infection with H37Rv.
variation in the experimental results. The age, sex or race of the donors may have affected the ability of immunomodulator-treated monocytes to kill *M. tuberculosis*. It has been shown that race is an important factor in antimycobacterial activity *in vitro*. Crowle and Elkins (1990) found that cultured macrophages from black American donors tended to be more permissive for tubercle bacilli than those from white American donors. No details were given by Denis (1991b) of the donors used in his experiments so it was not possible to accurately replicate this part of the procedure. However, in Leicester, donor A. was a twenty-nine-year-old, white male and donor B. was a twenty-six-year-old, white female.

Another potential source of variation between the experiments performed in the U.K. and those reported by Denis (1991b) was the strain of *M. tuberculosis* used to infect the monocytes. *Mycobacterium tuberculosis* H37Rv was employed in all experiments but it cannot be assumed that the cultures of H37Rv used in the Leicester, London and Quebec laboratories were completely identical.

It is interesting to note that neither Denis nor any other researchers have since published articles that employ the model of monocyte activation originally described in 1991 (Denis, 1991b). Perhaps it has been universally found that the activation of human monocytes with interferon-γ, tumour necrosis factor-α and calcitriol is not reliably reproducible.

### 3.1.2. The Antimycobacterial Activity of Human Macrophages

Following the failure to reproduce the data of Denis (1991b), the experiment described by Crowle and May (1981) was attempted. Again the method was as close as possible to that published. The monocytes of four donors (A-D) were isolated and used in the procedure. Each donor had previously been vaccinated with BCG. It was essential to collect blood from BCG-vaccinated individuals as Crowle and May (1981) found that only lymphocytes from immune subjects were capable of producing a supernatant that could subsequently activate macrophages for antimycobacterial activity.

Monocytes were incubated for seven days to allow maturation into macrophages. During this time homologous lymphocytes were stimulated with an antigen derived from *M. bovis* BCG. The supernatant collected from these cells was denoted “growth inhibition factor” (GIF). After the
seven day incubation period the macrophages were infected with *M. bovis* BCG for one hour at an
infection ratio of five bacteria to one macrophage. Extracellular mycobacteria were washed away
and the survival of the remaining bacteria was followed for another seven days by counting the
number of colony forming units on agar. GIF was used to activate half of the macrophage
monolayers and was added to the macrophages thirty minutes, two days and four days post-
infection (Crowle and May, 1981).

Immediately after the phagocytosis period (time zero), the numbers of monolayer-
associated mycobacteria were recorded (Table 3.5.). Analysis of the supernatant at time zero was
not deemed necessary as the infection ratio was just five bacilli to one macrophage and the
monolayer was washed six times after the infection period. It was assumed that the vast majority
of extracellular bacteria was removed by these washings.

As \(2.5 \times 10^5\) *M. bovis* BCG bacilli were initially added to each well, it can be calculated that
an average of 11.49% \(\pm\) 6.86 (range 6.05-21.48%) of the mycobacterial inoculum was taken up
by the macrophage monolayers over the one hour infection period (Table 3.5.). It is possible that
a greater number of *M. bovis* BCG were taken up by the macrophages than was observed and that a
certain number of the phagocytosed mycobacteria were killed during the hour. Indeed, Crowle
(1988) reports that one half of the total number of viable bacilli phagocytosed by macrophages
are killed as they are ingested.

Just one set of wells could be sampled at day 0 (Table 3.5.) because GIF was first added to
the macrophage monolayers thirty minutes after the end of the phagocytosis period. Up to this
point all wells were identical. It was found that neither GIF-treated nor untreated macrophages
were able to inhibit the growth of *M. bovis* BCG over the seven day incubation period (Table 3.5.).
In the four experiments, the total number of bacteria in the wells of treated monolayers increased
by an average of 1.39 \(\pm\) 0.44 log (range 0.75-1.73) while the total number of bacteria in the
wells of untreated monolayers increased by an average of 1.43 \(\pm\) 0.41 log (range 0.82-1.67).
These results are in contrast to those of Crowle and May (1981) who found that macrophages
treated with GIF, but not untreated macrophages, were able to control the intracellular
replication of *M. bovis* BCG over seven days. Thus, *M. bovis* BCG was able to replicate within
untreated macrophages for an average four-fold increase in a seven day infection period.
Table 3.5. The Fate of *M. bovis* BCG in Human Macrophages Measured by Colony Counting (Donors A-D.).

<table>
<thead>
<tr>
<th></th>
<th>DAY 0</th>
<th>DAY 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MEAN log&lt;sub&gt;10&lt;/sub&gt; CFU ± S.D.</td>
<td>TREATED&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Monolayer associated</td>
<td>5.18 ± 0.05</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer supernatant</td>
</tr>
<tr>
<td>B. Monolayer associated</td>
<td>5.32 ± 0.08</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer supernatant</td>
</tr>
<tr>
<td>C. Monolayer associated</td>
<td>5.40 ± 0.16</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer supernatant</td>
</tr>
<tr>
<td>D. Monolayer associated</td>
<td>5.73 ± 0.12</td>
<td>Total</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer associated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Monolayer supernatant</td>
</tr>
</tbody>
</table>

The results show the total number of bacterial colony forming units (CFU) in the well, the number of monolayer-associated bacteria and the number of bacilli recovered from the monolayer supernatant. All values represent the mean ± S.D. of at least three wells.

<sup>a</sup>100μl of Growth Inhibition Factor were added 30 minutes, 2 days and 4 days after the infection with BCG.
However, this intracellular replication was suppressed in GIF-treated macrophages (Crowle and May, 1981).

GIF was not toxic to the macrophages. There was a mean 31.00% ± 2.58 (range 28-34%) loss of cells from the treated monolayers and a mean 30.50% ± 1.91 (range 28-32%) loss of cells from the untreated monolayers over the seven days (Table 3.6.). It is likely that this destruction of the macrophage monolayer was due to the detachment of heavily infected cells (Rook, 1990). The large numbers of mycobacteria present in the supernatant after seven days incubation (Table 3.5.) probably originated from these heavily infected cells.

It is difficult to say why inhibition of mycobacterial replication was not observed. There was a difference between my experiments and those of Crowle and May in the way antimycobacterial activity was assessed. Crowle and May (1981) used acid-fast staining and [³H] thymidine incorporation to measure stasis of the bacteria. The acid-fast stain allowed a visual assessment of antimycobacterial activity. For example, Crowle and May observed that untreated macrophages contained huge numbers of mycobacteria but that GIF-treated macrophages possessed very few intact mycobacteria. However, it was found by Crowle and May (1981) that the method of [³H] thymidine incorporation was not reliable as an assay of antimycobacterial activity because the replicating tubercle bacilli did not incorporate measurable quantities of [³H] thymidine.

There are disadvantages to both of the assays used by Crowle and May (1981). Acid-fast staining cannot distinguish between viable and non-viable mycobacteria and can therefore give an inaccurate measure of antimycobacterial activity. The method of quantitating [³H] thymidine incorporation into bacterial DNA cannot distinguish bactericidal activity from bacteriostatic activity, as it is not possible to ascertain if a decrease in uptake of [³H] thymidine by mycobacteria is due to bacteriostasis or due to bacterial death. Rook et al (1985) have described identical problems when measuring the incorporation of [³H] uracil into mycobacterial RNA. It was therefore decided to use the viable count assay in my experiments. This technique only measures viable bacteria and can distinguish bacteriostasis from bactericidal activity. Thus, the difference between the two sets of experiments in the way antimycobacterial activity was measured should not have affected the final result.
Table 3.6. The DNA Content of the Macrophage Monolayer (Donors A.-D.).

<table>
<thead>
<tr>
<th>MEAN µg DNA/MONOLAYER ± S.D.</th>
<th>DAY 0</th>
<th>DAY 7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TREATED&lt;br&gt; A. 2.91 ± 0.170</td>
<td>1.92 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>B. 3.45 ± 0.031</td>
<td>2.43 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>C. 3.69 ± 0.006</td>
<td>2.67 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>D. 3.34 ± 0.135</td>
<td>2.26 ± 0.05</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

*100µl of Growth Inhibition Factor were added 30 minutes, 2 days and 4 days after the infection with BCG.
There are a number of alterations that could be made to the experimental technique which may increase the ability of human macrophages to demonstrate antmycobacterial activity in vitro. Macrophages could be activated before the infection with M. bovis BCG by the addition of GIF during the maturation period. Alternatively, a larger amount of GIF could be added to the monolayers or the GIF could be administered more often. A lower infection ratio could be used, which would also have the advantage of minimising the destruction of the monolayer through loss of heavily infected cells. Alterations could be made in the length of time the monocytes spend in culture before the infection period. Douvas et al. (1986) have reported that M. tuberculosis Erdman grew readily in freshly isolated blood monocytes and in monocyte-derived macrophages obtained after seven days in culture. However, monocytes cultured for only three days prior to infection were less permissive for the mycobacteria (Douvas et al., 1986). These variations in the procedure could be attempted if the experiment of Crowle and May (1981) is repeated in the future.

The experiment described by Crowle and May (1981) is technically difficult and there are numerous points, for example the maturation of monocytes into macrophages or the infection of the monolayers, at which the procedure may go wrong. The production of antigen from M. bovis BCG and the generation of GIF from stimulated lymphocytes are the two steps in the experimental procedure which are particularly crucial. If the antigen is faulty, then the lymphocytes will not be correctly stimulated and the composition of the GIF will be altered. If the lymphocytes are not under optimal conditions or are not fully viable, then the composition of the GIF will again be affected. In these circumstances, activation of macrophages for antmycobacterial activity will not occur. Perhaps experimental error was therefore responsible for the failure to observe killing or inhibition of M. bovis BCG in my experiments (Table 3.5.). An indication of the difficulty of the experiment (Crowle and May, 1981) is given by the fact that Crowle and May themselves have failed to employ the technique in more recent publications, preferring to use defined cytokines to activate human macrophages for inhibition of mycobacterial replication (for example see Crowle et al., 1987; Crowle and Ross, 1988; Crowle and Elkins, 1990; Crowle et al., 1990). These experiments could be repeated if a model of macrophage antmycobacterial activity is required in the future.
3.1.3. Demonstrating Human Macrophage Antimycobacterial Activity in vitro.

Until a culture system is devised in which human macrophages can be shown to reproducibly kill *M. tuberculosis*, it will be difficult to study macrophage tuberculocidal mechanisms. Just why it is proving so difficult to activate human macrophages *in vitro* for tuberculocidal activity is not clear. Cultured human macrophages can be activated to kill many other facultative intracellular pathogens (for example see Douvas et al, 1985; Byrne and Carlin, 1989; Murray et al, 1989b; Byrd and Horwitz, 1990) and the activation process has been demonstrated to be quite a simple procedure. It often involves the addition of just one cytokine, such as interferon-γ. Possibly, the right combination or concentration of cytokines that activate cultured human macrophages for tuberculocidal activity has not yet been tested. The order and timing of the addition of cytokines to macrophages may also be important for the development of antimycobacterial properties (section 1.8.). In addition to cytokines, it is likely that immunologically active vitamins, such as vitamin D₃ (Rook et al, 1986b) and retinoic acid (Crowle and Ross, 1989), are involved in the activation of tuberculocidal activity in human macrophages. However, *M. tuberculosis* has a refractory nature and macrophages may need to be highly activated to have any effect on the bacillus. The complex nature of macrophage activation for tuberculocidal activity may mean that it is not possible to reach this high level of activation *in vitro*.

Adding purified cytokines and/or immunologically active vitamins to macrophages may be the wrong approach. Sypek et al (1993) have shown that BCG-sensitised lymphocytes can induce antimycobacterial activity in cultured, BCG-infected murine macrophages. This effect required direct contact between the two cells and was not due to the secretion of cytokines by the lymphocytes. It was postulated that tumour necrosis factor-α, which was associated with the lymphocyte membrane, may have been responsible for activation of the macrophages. Thus, contact between T-cells and macrophages as well as the addition of soluble cytokines may be necessary for a higher degree of macrophage activation *in vitro*.

Alternatively, human macrophages may express poor antimycobacterial activity *in vitro* because the conditions in culture do not reproduce the *in vivo* conditions. One important aspect to consider is the availability of oxygen. It has been reported that *M. tuberculosis* has a reduced...
intracellular growth rate in human macrophages cultured at physiological oxygen concentration (5%) compared to macrophages cultured at ambient oxygen concentration (20%) (Meylan et al., 1992). However, the growth of *M. tuberculosis* in medium alone was not affected by the concentration of oxygen (Meylan et al., 1992).

The mechanism of the reduced growth of tubercle bacilli in macrophages at the low oxygen concentration was not ascertained. Possibly, the intracellular oxygen concentration in macrophages cultured at the low oxygen concentration dropped to a level that affected the mycobacteria (Meylan et al., 1992). Alternatively, the macrophages at the low oxygen concentration may have had more effective tuberculocidal mechanisms. Indeed, macrophages cultured at the low oxygen concentration displayed enhanced superoxide anion production on stimulation compared to those macrophages cultured at the high oxygen concentration (Meylan et al., 1992). However, it is known that certain antimycobacterial mechanisms, such as the generation of aminoaldehydes by polyamine oxidase (section 1.9.2.), are less effective when tubercle bacilli have slow growth rates. The mechanism of aminoaldehyde toxicity is believed to be inhibition of nucleic acid synthesis and consequently protein synthesis. Aminoaldehydes exert maximum tuberculocidal activity on bacteria that have a high multiplication rate and are thus undergoing a high rate of nucleic acid synthesis. It can therefore be concluded that experiments that involve culturing macrophages at ambient oxygen concentration may be inaccurate as they do not reflect *in vivo* conditions.

Most experiments that attempt to show tuberculocidal activity in human cells use blood monocytes or monocyte-derived macrophages (for example see Douvas et al., 1986; Rook et al., 1986a; Robertson and Andrew, 1991; Carvalho de Sousa and Rastogi, 1992). As TB is primarily a disease of the lung, perhaps it would be more appropriate to use alveolar macrophages when studying the interaction between macrophages and *M. tuberculosis*. For example, human alveolar macrophages were shown to inhibit the replication of *M. tuberculosis* H37Ra to a greater extent than blood monocytes (Hirsch et al., 1994). It has also been demonstrated that liver and spleen tissue can efficiently overcome mycobacterial infection (Collins, 1990; North and Izzo, 1993). Macrophages from these sites should be investigated for tuberculocidal activity too.

Evaluating the cytokine response at the site of mycobacterial disease *in vivo* may lead to an
insight into which cytokines can activate human macrophages for antimycobacterial activity in vitro. Studies with leprosy patients (Yamamura et al., 1991) indicate that mRNA for interferon-γ and interleukin-2 is high in people with the tuberculoid form of the disease (section 1.3.). Expression of interleukin-4, interleukin-5 and interleukin-10 mRNA predominates in lepromatous leprosy patients (section 1.3.) (Yamamura et al., 1991). The TH1 type cytokines (section 1.5.) therefore appear to be important for protective immunity against M. leprae in vivo.

It has been widely reported that PPD-stimulated peripheral blood mononuclear cells harvested from TB patients release lower levels of interferon-γ (Onwubalili et al., 1985; Vilcek et al., 1986; Sanchez et al., 1994) and interleukin-2 (Toossi et al., 1986; Sanchez et al., 1994) and higher levels of interleukin-1 (Fujiwara et al., 1986) and interleukin-4 (Sanchez et al., 1994) than cells from healthy donors. Schauf et al. (1993) investigated the expression of cytokine mRNA in non-stimulated peripheral blood mononuclear cells of healthy donors and TB patients. The cells of healthy donors did not express mRNA for any cytokine. However, in the cells of TB patients, mRNA for interleukin-1, interleukin-8 and tumour necrosis factor-α was uniformly expressed. Most patients cells demonstrated interleukin-4 mRNA, seven of eighteen patients expressed interferon-γ mRNA but none expressed interleukin-2 mRNA. Zhang et al. (1995) reported that the peripheral blood monocytes of tuberculosis patients displayed a diminished production and a reduced mRNA expression of interferon-γ and interleukin-2 when stimulated with M. tuberculosis. However, there was no change in the production or mRNA expression for the TH2 cytokines; interleukin-4, interleukin-10 and interleukin-13. Identical results were obtained with purified T-cells (Zhang et al., 1995). Interestingly, it was found that antituberculosis therapy led to the recovery of the TH1 response. Thus, it would appear that people suffering from tuberculosis disease exhibit a diminished TH1 immune response.

Tuberculous pleuritis usually resolves without chemotherapy (Roper and Waring, 1955) which suggests that, unlike classical tuberculosis, cell-mediated immune responses result in effective clearance of the bacilli. Pleuritis therefore provides a model to study protective immune mechanisms against M. tuberculosis in vivo. Increased levels of vitamin D3 have been found in the pleural fluid of pleuritis patients (Barnes et al., 1989). Enhanced expression of mRNA for
interferon-γ and interleukin-2 (Barnes et al, 1993b), decreased expression of interleukin-4 mRNA (Barnes et al, 1993b) and an increased concentration of tumour necrosis factor-α (Barnes et al, 1990) was observed in the pleural fluid of patients with tuberculous pleuritis. Concentrations of interleukin-10 were also elevated in the pleural fluid (Barnes et al, 1993b) which may have served to down-regulate the immune response to *M. tuberculosis* in vivo. Thus, a large body of evidence has been collected that indicates that the TH₁ type cytokines are involved in effective immunity against mycobacterial infection. It must be concluded that these TH₁ cytokines would be most likely to be involved in the activation of macrophages for tuberculocidal activity *in vitro*.

Guinea pigs constitute an accurate animal model for human tuberculosis (section 1.8.2.). As an alternative to working with human cells or TB patients, experiments that have previously been performed with mice could be repeated and expanded with guinea pigs. In this way, evidence may be gained as to which cytokines play an important role in guinea pig antimycobacterial activity. It might then be possible to use these cytokines to activate human macrophages to kill or inhibit *M. tuberculosis* *in vitro*.

In one set of experiments, recombinant cytokines were given to mice that had been infected with mycobacteria. Denis (1991d) reported that continuous infusion of interferon-γ led to increased resistance against a lethal dose of *M. tuberculosis* and to decreased growth of bacteria in the spleen, liver and lung. In contrast, Flynn et al (1995) have found that Interferon-γ has no effect on the survival of mice infected with *M. tuberculosis* or on the bacterial burden. Jeevan and Asherson (1988) observed that *in vivo* administration of interleukin-2 significantly reduced the bacterial counts in spleens of *M. bovis* BCG-infected mice. Administration of interleukin-12 to mice infected with *M. tuberculosis* was observed to result in increased survival times and a decreased bacterial burden (Flynn et al, 1995). Infusion of tumour necrosis factor-α was reported to increase the resistance of mice to *M. bovis* BCG (Kindler et al, 1989) and to *M. tuberculosis* (Denis, 1991d).

An alternative approach has been to use antibodies against cytokines. Depletion of interferon-γ (Denis, 1991d) or tumour necrosis factor-α (Kindler et al, 1989; Denis, 1991d) by antibodies enhanced the susceptibility of mice to *M. tuberculosis* (Denis, 1991d) and *M. bovis*.
Experiments have also been performed with mice that have been genetically altered. Thus, mice have been created with a disruption in the gene coding for interferon-γ (Flynn et al., 1993) and with a disrupted gene for the interferon-γ receptor (Kamijo et al., 1993). Upon infection with *M. tuberculosis* (Flynn et al., 1993) or *M. bovis* BCG (Kamijo et al., 1993), these mice were unable to restrict the growth of the bacilli and died.

A study was undertaken by Yoshida and colleagues (1995) of two strains of mice (BALB/c and B10) that both express the susceptible allele of the Bcg gene, Bcg<sup>®</sup> (section 1.5.). Vaccination with *M. bovis* BCG was demonstrated to lead to acquired protective immunity against a subsequent BCG infection in B10 mice but not in BALB/c mice. On infection with BCG, a high level of expression of interferon-γ, interleukin-2 mRNA and interleukin-12 mRNA in the spleens of B10 mice compared to the spleens of BALB/c mice was measured (Yoshida et al., 1995). However, expression of tumour necrosis factor-α was comparable in both mouse strains (Yoshida et al., 1995).

Pulmonary granulomas have been induced in mice by the injection of live *M. bovis* BCG, followed two weeks later by an injection of PPD-coated Sepharose beads (Chensue et al., 1994). It was found that the associated T-cell response was of a TH<sub>1</sub> nature (Chensue et al., 1994).

All these experiments have provided evidence for the importance of the TH<sub>1</sub> cytokines (interferon-γ, interleukin-2 and interleukin-12) and tumour necrosis factor-α in murine antimycobacterial activity. It has previously been demonstrated that interferon-γ and tumour necrosis factor-α can synergistically activate murine macrophages *in vitro* to inhibit (Flesch and Kaufmann, 1990a) or kill (Chan et al., 1992) *M. tuberculosis*.

Thus, all the evidence from murine and human studies points to the TH<sub>1</sub> type cytokines being involved in the protective immune response to *M. tuberculosis*. However, Crowle et al. (1990) have previously reported that a combination of interferon-γ and interleukin-2 failed to activate human monocyte-derived macrophages to suppress the growth of virulent tubercle bacilli *in vitro*. Possibly, positive results would have been obtained with these cytokines if attempts had been made to mimic *in vivo* conditions. Thus, human alveolar macrophages could be used instead of monocyte-derived macrophages, the concentration of oxygen could be decreased to
physiological levels or T-cells could be included in the wells with macrophages.

Most importantly, a systematic approach should be made towards achieving activation of human macrophages for tuberculocidal activity. The type of cell, the handling of cells following isolation, the conditions of culture, the strain of mycobacterium and the infection ratio that is to be used in experiments should be agreed upon. All possible combinations of the TH1 type cytokines and the immunologically active vitamins (section 1.8.) should be tested and variations should be made in their concentration and in the sequence and timing of their addition to cells. Ideally, all macrophages should be harvested from one person to eliminate the variation that is experienced between donors (Crowle and May, 1981; Rook et al, 1986a). In this way it may be possible to discover the procedure that allows activation of human macrophages for tuberculocidal activity.

3.2. The Antimycobacterial Activity of Guiney Pig Macrophage Lysosomes.

It is not yet known if lysosomes have a role to play in the antimycobacterial activity of macrophages. Mycobacteria have been shown to be resistant to the action of lysosomal enzymes, both in cell-free systems (Myrvik et al, 1953a and 1953b; Weiss and Singer, 1953; Stahelin et al, 1956; Ginsburg, 1979; Lowrie and Andrew, 1988) and in cultured, non-activated murine macrophages (Armstrong and D'Arcy Hart, 1975). It is surprising therefore that mycobacteria have been observed to avoid exposure to the lysosomal enzymes. *Mycobacterium tuberculosis* and *M. bovis* BCG were reported to inhibit phagosome-lysosome fusion in non-activated murine (Armstrong and D'Arcy Hart, 1971 and 1975) and human (Clemens and Horwitz, 1995) macrophages. In contrast, McDonough et al (1993) reported that phagolysosomes were formed inside non-activated human and murine macrophages soon after infection but that *M. tuberculosis* had the capacity to later escape from these phagolysosomes.

Phagosome-lysosome fusion can be induced by activating macrophages with cytokines. Increased phagosome-lysosome fusion was found to occur when human macrophages were incubated with macrophage colony stimulating factor before infection with *M. avium* (Duzguenes et al, 1993) and when murine macrophages were incubated with interferon-γ before infection with *M. leprae* (Sibley et al, 1987). It was not reported whether the lysosomal contents of the
activated human macrophages were toxic to *M. avium* (Duzgunes *et al.*, 1993). However, Sibley *et al* (1987) found that increased phagosome-lysosome fusion was associated with an increase in the number of fragmented and damaged bacilli, indicating that the lysosomal contents of activated murine macrophages are antimycobacterial.

Guinea pig alveolar macrophages can be activated to kill *M. tuberculosis* in vitro by employing an *in vivo* vaccination protocol (O'Brien and Andrew, 1991; O'Brien *et al.*, 1991). In order to have an insight into the role lysosomal constituents may have in guinea pig macrophage tuberculocidal activity, the specific activities of lysosomal enzymes and the antimycobacterial properties of isolated lysosomes were tested.

### 3.2.1. The Specific Activities of Lysosomal Enzymes in Guinea Pig Macrophage Homogenates.

Dannenberg (1989) has reported that activated macrophages in the centre of tuberculous lesions exert a high level of antimycobacterial activity and that, histochemically, these cells are abundant in lysosomal enzymes. Thus, macrophages from BCG-vaccinated guinea pigs may possess an increased lysosomal enzyme content that allows effective killing of *M. tuberculosis* in vitro. Analysis of lysosomal enzyme activity had already been undertaken with macrophages from BCG-vaccinated guinea pigs and a limited number of non-vaccinated guinea pigs (O'Brien, 1992). In this thesis, the specific activities of lysosomal enzymes in macrophages from non-vaccinated guinea pigs were determined to complete the study of O'Brien (1992). Alveolar macrophages were disrupted in iso-osmotic sucrose, the nuclei were removed by centrifugation and the activities of lysosomal enzymes in the resulting post-nuclear supernatant were measured. The combined results are shown in Table 3.7. None of the tested enzymes had significantly different specific activities in macrophages from vaccinated and non-vaccinated animals, except catalase (*P* < 0.001). This enzyme had a six-fold greater specific activity in post-nuclear supernatants from vaccinated guinea pigs compared to non-vaccinated guinea pigs. Unfortunately, catalase is believed to be located in peroxisomes and is not thought to be a constituent of the lysosome (Jackett *et al.*, 1980).

It can be hypothesised that the increase in the activity of catalase is a protective response to the greater oxidative activity of BCG-induced macrophages (Jackett *et al.*, 1981b). However,
Table 3.7. Enzyme Activity of Post-nuclear Supernatants.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Non-vaccinated Guinea pig</th>
<th>Vaccinated Guinea pig</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>1.49 ± 0.4 (4)</td>
<td>1.79 ± 0.2 (4)</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>2.98 ± 1.1 (4)</td>
<td>1.82 ± 0.3 (4)</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>0.56 ± 0.2 (4)</td>
<td>0.30 ± 0.1 (4)</td>
</tr>
<tr>
<td>Catalase</td>
<td>0.30 ± 0.0 (4)</td>
<td>1.87 ± 1.0 (3)</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>0.24 ± 0.1 (4)</td>
<td>0.80 ± 0.5 (3)</td>
</tr>
</tbody>
</table>

All the experiments with vaccinated guinea pigs and half the experiments with non-vaccinated guinea pigs were performed by S. O'Brien (1992). The results are mean values ± S.D. The numbers of homogenates tested for each enzyme are shown in parantheses.
increased catalase content could have adverse effects for the cell in that it may also protect the phagocytosed mycobacteria from the respiratory burst. Alternatively, catalase may contribute to the antimicrobial process because, under certain conditions, catalase can act as a peroxidase (Jackett et al., 1980). Peroxidase incorporates halide ions into hydrogen peroxide, forming extremely toxic acids (Jackett et al., 1980). It has been reported that catalase enters the phagosome during phagocytosis (Stossel et al., 1972; D'Arcy Hart and Armstrong, 1974). Catalase could therefore be involved in the tuberculocidal activity of activated macrophages.

There is no evidence for a general change in the activity of lysosomal enzymes on guinea pig macrophage activation (Table 3.7.). In these experiments the more prominent lysosomal enzymes were measured. It is of course possible that the specific activities of other lysosomal enzymes increased when guinea pig macrophages became activated.

In contrast to the results reported here (Table 3.7.), Chandrasekhar and Mukherjee (1990) found that BCG vaccination increased the activity of β-galactosidase in guinea pig alveolar macrophages. However, the activities of N-acetyl-β-glucosaminidase and lysozyme remained unaltered (Chandrasekhar and Mukherjee, 1990). There have also been reports of the variable effect of BCG vaccination on lysosomal enzyme activity in the rabbit. The specific activities of N-acetyl-β-glucosaminidase and β-galactosidase were found not to increase in alveolar macrophages after BCG vaccination (Pilatte et al., 1987). However, it was reported that the specific activities of β-glucuronidase and lysozyme increased in BCG-vaccinated rabbit alveolar macrophages (Sorber et al., 1973). In a third study with rabbit alveolar macrophages, it was found that N-acetyl-β-glucosaminidase and lysozyme activity decreased, β-galactosidase activity did not change and β-glucuronidase activity increased after BCG vaccination (Lowrie et al., 1979). Thus, there are conflicting data on the effect of BCG vaccination on lysosomal enzyme activity in rabbit alveolar macrophages.

3.2.2. The Antimycobacterial Activity of the Lysosomal Contents from Guinea Pig Macrophages.

In addition to measuring the specific activities of lysosomal enzymes, the lysosomal contents were tested for antimycobacterial activity in vitro. Initially, analytical isopycnic ultracentrifugation of the macrophage post-nuclear supernatant was performed with a linear
sucrose density gradient. This allowed the organelles present in the post-nuclear supernatant to be separated and the densities at which these subcellular organelles sedimented to be determined. It had been previously found that there was a high frequency of organelle breakage when the macrophages were continuously passaged through the 25 gauge needle during the process of homogenisation (O'Brien, 1992). The enzyme activity that was released from these broken organelles did not sediment into the gradient which made the enzyme assays designed to locate the organelles less effective. However, when care was taken to minimise organelle breakage, by harvesting the post-nuclear supernatant after every three passages, less than 10% of the total enzyme activity was found at the start of the gradient.

To determine the distribution of organelles within the continuous gradient, marker enzymes were assayed. The comparative activity of the enzymes within the gradient indicated the density to which lysosomes and other subcellular organelles sedimented. Marker enzymes were similarly used in studies with rabbit alveolar macrophages (Lowrie et al., 1979; Andrew et al., 1983), murine peritoneal macrophages (Canonico et al., 1978) and human neutrophils (Kane and Peters, 1975; Segal and Peters, 1977). The results are shown in Table 3.8.

On the basis of modal density, it can be seen that non-activated macrophages possessed two types of acid hydrolase-containing granules. One has a modal density of 1.19g cm\(^{-3}\) and the other has a modal density of 1.23g cm\(^{-3}\) (Table 3.8.). However, the activity of lysozyme peaked at 1.22g cm\(^{-3}\), indicating that lysozyme was only present in the more dense lysosomal population. Three hydrolase-containing granule types have been observed in non-activated rabbit alveolar macrophages (Lowrie et al., 1979). Lysozyme was found to be present in a distinct lysosome population and possibly in a set of granules containing acid hydrolases (Lowrie et al., 1979).

A different distribution of lysosomal enzymes was seen with BCG-induced guinea pig macrophages (Table 3.8.). All of the lysosomal enzymes tested showed a single peak of activity at 1.21g cm\(^{-3}\). The effect of BCG-vaccination, therefore, was to reduce the heterogeneity of lysosomes in guinea pig alveolar macrophages. BCG vaccination has also been shown to influence the properties of the lysosomal enzyme-containing granules in rabbit alveolar macrophages (Sorber et al., 1973; Lowrie et al., 1979). An increase in modal density of these granules was observed after vaccination.
Table 3.8. The Modal Densities of Subcellular Organelles in Activated and Non-activated Macrophages.

<table>
<thead>
<tr>
<th>Marker Enzyme</th>
<th>Subcellular Location</th>
<th>Activated Macrophage</th>
<th>Non-activated Macrophage</th>
</tr>
</thead>
<tbody>
<tr>
<td>5'nucleotidase</td>
<td>Plasma membrane</td>
<td>1.15</td>
<td>1.16</td>
</tr>
<tr>
<td>Neutral-α-glucosidase</td>
<td>Endoplasmic reticulum</td>
<td>1.15</td>
<td>1.16</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>Mitochondria</td>
<td>1.21</td>
<td>1.21</td>
</tr>
<tr>
<td>N-acetyl-β-glucosaminidase</td>
<td>Lysosome</td>
<td>1.21</td>
<td>1.19, 1.23</td>
</tr>
<tr>
<td>β-galactosidase</td>
<td>Lysosome</td>
<td>1.21</td>
<td>1.19, 1.23</td>
</tr>
<tr>
<td>β-glucuronidase</td>
<td>Lysosome</td>
<td>1.21</td>
<td>1.19, 1.23</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>Lysosome</td>
<td>1.21</td>
<td>1.22</td>
</tr>
<tr>
<td>Catalase</td>
<td>Peroxisome</td>
<td>1.21</td>
<td>1.23</td>
</tr>
</tbody>
</table>
Once the densities at which the subcellular organelles sedimented were known (Table 3.8.), it was possible to construct a preparative, discontinuous sucrose gradient to enable the lysosomes to be separated from the plasma membrane and endoplasmic reticulum. On the basis of the data from analytical ultracentrifugation (Table 3.8.), sucrose solutions of densities 1.30, 1.18 and 1.13 g cm\(^{-3}\) were chosen for use in the preparative gradient. Lysosomes would be isolated at the boundary of densities 1.18 and 1.30 g cm\(^{-3}\) and would be separated from the endoplasmic reticulum and plasma membrane which sediment to approximately 1.15 g cm\(^{-3}\). Malate dehydrogenase and catalase also showed peaks of activity around 1.20 g cm\(^{-3}\) (Table 3.8.) which would lead to mitochondria and peroxisomes sedimenting with the lysosomes. It was therefore accepted that any isolated lysosomal sample would be contaminated with these non-lysosomal components.

Following centrifugation of preparative gradients, selected enzymes were assayed to ensure that the lysosomal and non-lysosomal components appeared on the gradient at the anticipated densities. It was found that of the total activity present within the gradient, 93% of N-acetyl-β-glucosaminidase activity and 81% of lysozyme activity occurred at a density of 1.18 g cm\(^{-3}\). 80% of the total activity of neutral-α-glucosidase was present at 1.13 g cm\(^{-3}\) whilst 72% of the total activity of malate dehydrogenase occurred at 1.18 g cm\(^{-3}\). 5’nucleotidase was found not to have entered the gradient. Thus, by performing preparative ultracentrifugation, it was possible to isolate fractions enriched for lysosomes, at a density of 1.18 g cm\(^{-3}\).

It had been previously determined that guinea pig alveolar macrophage homogenates possessed a low level of antibacterial activity (O'Brien, 1992). Efforts were therefore made to concentrate the lysosomal constituents in the fractions obtained from the preparative gradient by the method of acid extraction. The technique of acid extraction selects for acid soluble factors. Acid may exert a solubilising effect by inducing a proteolytic event, by inducing a conformational change that disrupts the association of antibacterial factors with membranes and subcellular organelles or by mediating the displacement of charge interaction between antibacterial factors and cell membranes or subcellular organelles. Acid extraction was shown to be successful in releasing and enriching bactericidal factors in lysosomes from human neutrophils (Gabay et al, 1986) and guinea pig alveolar macrophages (O'Brien, 1992). Acid-extracted lysosomal fractions
were capable of killing strains of *Staphylococcus aureus, Streptococcus pneumoniae, Pseudomonas aeruginosa, Listeria monocytogenes* and *Escherichia coli* (Gabay et al, 1986; O'Brien, 1992).

The antimycobacterial activity of the acid-extracted lysosomal fraction of guinea pig macrophage homogenates is shown in Table 3.9. A limited number of acid-extracted lysosomal fractions from vaccinated guinea pigs had previously been tested by O'Brien (1992). *Mycobacterium tuberculosis* H37Rv was incubated in the fractions at a protein concentration of 300μg ml⁻¹ for up to twenty-four hours. The concentration of protein that was used was based on observations by Gabay et al (1986). They found that the antibacterial activity of human neutrophil extracts was maximal at 300μg protein ml⁻¹.

Only the extracts from lysosomes of macrophages from vaccinated guinea pigs possessed significant (P< 0.001) tuberculocidal activity (Table 3.9.). Lysosomal contents from activated macrophages were capable of killing an average of 40% (range 24-54%) of *M. tuberculosis* H37Rv over two hours. In contrast, there was no significant change in mycobacterial numbers in the lysosomal contents from non-activated macrophages. The antimycobacterial effects of the lysosomal contents from vaccinated guinea pigs seen at two hours was not increased by extending the assay to twenty-four hours and killing was still significant after twenty-four hours (P< 0.01). Citrate buffer alone showed no killing effect.

Tuberculocidal activity was tested with two separate buffers at two different pH values. Citrate buffer, pH5.5, was employed because Gabay et al (1986) had found optimum killing of bacteria with this buffer at this pH. Unfortunately, citrate does not buffer at pH7 so phosphate buffer was used instead. The activity against *M. tuberculosis* H37Rv was found to be pH-dependent (Table 3.9.). Thus, there was no significant change in mycobacterial numbers when incubated for up to twenty-four hours in lysosomal extracts from macrophages of vaccinated or non-vaccinated animals at pH7. In future experiments, it would be useful to test the antimycobacterial effects of the lysosomal contents in phosphate buffer, pH5.5. This would allow a more accurate comparison of the effect pH has on the activity of lysosomal extracts.

The fact that the acid-extracted lysosomal fractions were active at pH5.5 in vitro is consistent with the tuberculocidal factors being active in the acidic environment of the macrophage phagolysosome (de Duve et al, 1978). However, in non-activated macrophages,
Table 3.9. Activity of Lysosomes from Vaccinated and Non-vaccinated Guinea Pigs against *M. tuberculosis* H37Rv.

<table>
<thead>
<tr>
<th>Lysosomal Fraction(^a)</th>
<th>CITRATE BUFFER (pH5.5)</th>
<th>PHOSPHATE BUFFER (pH7.0)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean % change in CFU ml(^{-1}) ((T_0-T_2))</td>
<td>Mean % change in CFU ml(^{-1}) ((T_0-T_{24}))</td>
</tr>
<tr>
<td>Activated</td>
<td>-40.00 ± 12.66 ((6)^c)</td>
<td>-38.25 ± 12.77 ((4)^c)</td>
</tr>
<tr>
<td>Heated Activated</td>
<td>-13.50 ± 14.43 ((4)^c)</td>
<td>ND</td>
</tr>
<tr>
<td>Non-activated</td>
<td>1.75 ± 6.42 ((4))</td>
<td>4.25 ± 5.93 ((4))</td>
</tr>
<tr>
<td>Heated Non-activated</td>
<td>-4.50 ± 0.87 ((4))</td>
<td>ND</td>
</tr>
<tr>
<td>Control (SVEH)</td>
<td>1.00 ± 12.86 ((6)^c)</td>
<td>5.25 ± 8.50 ((4))</td>
</tr>
<tr>
<td>Heated Control</td>
<td>0.50 ± 2.50 ((4))</td>
<td>ND</td>
</tr>
<tr>
<td>Buffer</td>
<td>-0.33 ± 4.57 ((6)^c)</td>
<td>2.50 ((2))</td>
</tr>
</tbody>
</table>

ND: Not Done

The values in parantheses are the number of experiments done. Each experiment is data collected from the macrophages of an individual guinea pig.

\(^a\)Lysosomal fractions were termed "activated" when they were obtained from BCG-vaccinated guinea pigs and "non-activated" when they were obtained from non-vaccinated guinea pigs.

\(^b\)A negative value represents a decrease in CFU ml\(^{-1}\).

\(^c\)Half of these replicate experiments were performed by S. O'Brien (1992).
Mycobacteria are capable of inhibiting the acidification of phagosomes. *Mycobacterium tuberculosis* has been observed inside non-acidic phagosomes (Crowle *et al*., 1991) and it has been reported that the phagosomal membrane surrounding the tubercle bacilli does not acquire subunits (Sturgill-Koszycki *et al*., 1994) or accessory proteins (Xu *et al*., 1994) of the proton pump required to acidify the phagosome. Whether mycobacteria can inhibit the process of acidification in activated macrophages has not been investigated. However, it can be concluded that if *M. tuberculosis* comes into contact with lysosomal constituents in an acidic environment inside the activated guinea pig macrophage, these constituents are capable of killing the bacterium.

Additional experiments need to be done for a more complete picture of the antimycobacterial activity of the lysosomal extracts. *Mycobacterium tuberculosis* could be incubated with the lysosomal contents over a range of protein concentration and a range of pH. This would determine the optimal conditions for lysosomal tuberculocidal activity. In addition, the ability of the lysosomal extracts to kill a number of strains of *M. tuberculosis* (section 2.5.) could be tested to ascertain if there is a relationship between the susceptibility of the strains and virulence.

The finding that there is a lack of tuberculocidal activity exhibited by lysosomal fractions from non-activated guinea pig macrophages supports the observations of Armstrong and D'Arcy Hart (1975). They found that intracellular replication of *M. tuberculosis* H37Rv, in non-activated murine peritoneal macrophages, was not inhibited by exposure to the lysosomal contents when bacteria were coated with antibody as a means of overcoming inhibition of phagosome-lysosome fusion.

The activity of acid-extracted lysosomal extracts from BCG-vaccinated guinea pigs could be due to an increase in the amount of the active substance(s). However, there was no increase in the specific activities of the lysosomal enzymes that were tested in these experiments (Table 3.7.). Alternatively, a qualitative alteration in the active substance(s) may make them tuberculocidal.

The antimycobacterial activity exhibited at pH 5.5 was partially heat sensitive. Boiling the lysosomal extracts from activated macrophages for fifteen minutes resulted in a significant (P< 0.05) reduction in antimycobacterial activity, although killing of *M. tuberculosis* H37Rv still
occurred (Table 3.9). In three experiments the tuberculocidal activity was reduced by an average of $12.33 \pm 2.31\%$, in the fourth experiment killing was completely abolished. Thus, the tuberculocidal properties of activated macrophages are likely to be due partly to heat labile factors and partly to heat stable factors which are present in the lysosomal extracts.

Heat lability suggests the involvement of antimycobacterial proteins and enzymes. For example, the acid hydrolases are heat labile (Barret and Heath, 1977). The identity of the heat stable tuberculocidal factor(s) is less clear. A possibility is lysozyme which is reported to be heat stable (Barret and Heath, 1977). However, lysozyme has been reported to only cause stasis of mycobacteria unless present in extremely large concentrations (Myrvik et al, 1953a and 1953b). Toxic fatty acids and lipids are also heat stable. Fatty acids have been shown previously to exert a strong and rapid mycobactericidal effect in low concentrations and at an acidic pH (Kondo and Kanai, 1972; Kanetsuna, 1985). Kochan and Golden (1973) observed that the inhibition of tubercle bacilli growth by lysates of alveolar macrophages from BCG-vaccinated guinea pigs was due to fatty acids. However, the antimycobacterial activity of liver cells collected from BCG-vaccinated guinea pigs was reported to be due to fatty acids associated with the cellular membrane and not with the lysosomal contents (Kochan et al, 1972). In addition, given the method of extraction used in this study, it is unlikely that toxic fatty acids or lipids explain the antimycobacterial activity seen here (Table 3.9.).

It is possible that catalase could have been contributing to the tuberculocidal activity of the acid-extracted lysosomal fractions from activated macrophages. There was increased catalase activity in macrophage homogenates from vaccinated guinea pigs (Table 3.7.) and it was found that catalase activity was associated with the lysosomal fractions (Table 3.8.), presumably because the lysosomes and peroxisomes were co-migrating through the gradient. As described earlier, catalase can act as a peroxidase, thereby enhancing the toxicity of hydrogen peroxide. However, peroxidase activity cannot be responsible for the tuberculocidal properties of the acid-extracted lysosomal fractions described here, as the hydrogen peroxide content of the post-nuclear supernatants from both activated and non-activated macrophages was found to be zero. In addition, the antimycobacterial activity exhibited at pH5.5 (Table 3.9.) by acid-extracted lysosomal fractions cannot be due to cationic proteins (section 1.9.2.) because these proteins are only active
at an alkaline or near neutral pH.

Further characterisation of the tuberculocidal factors present in the acid-extracted lysosomal fractions is required. Future experiments might include a crude analysis of the molecular weight by passing the lysosomal fractions through a filter before assaying for tuberculocidal activity. Also, the proteinaceous nature of the tuberculocidal factors could be determined by assessing the effect of proteolytic enzymes or protein denaturing substances, such as urea or sodium dodecyl sulphate, on the mycobactericidal activity. To ascertain whether fatty acids or lipids really are contributing to the tuberculocidal activity, the preparation could be extracted with organic solvents prior to incubation with *M. tuberculosis*. Two antimycobacterial factors have been identified in crude lysosomal components obtained from lung and spleen homogenates of BCG-vaccinated mice (Kanai and Kondo, 1969b). One was a non-membrane-associated lipid and the other was a low molecular mass, protein species which was possibly membrane-associated.

Iron-binding proteins (section 1.9.2.) are possible candidates for the antimycobacterial factors found in acid-extracted lysosomal fractions. Evidence to support or refute this hypothesis could be gained by adding iron to the assay buffer before testing for tuberculocidal activity. A reduction in the amount of killing of *M. tuberculosis* in the presence of iron would indicate that iron-binding proteins were active in the lysosomal fractions. However, Kochan et al (1972) have reported that the antimycobacterial effect in lysosomal extracts from BCG-vaccinated guinea pigs was not affected by supplements of iron, indicating that iron-binding proteins are not lysosomal tuberculocidal factors.

In this study, the lysosomal contents of macrophages have been implicated in the tuberculocidal activity of BCG-vaccinated guinea pigs. Similar results have previously been obtained by Kanai and Kondo (1969a) who demonstrated antimycobacterial activity in whole lung homogenates from BCG-vaccinated guinea pigs. An acid-extracted, lysosomal-rich fraction was observed to kill *M. tuberculosis* H37Rv. This activity was shown to be acid-dependent and fully heat stable (Kanai and Kondo, 1969a), although the samples were only boiled for five minutes rather than the fifteen minutes used here.

Chandrasekhar and Mukherjee (1990) have provided evidence for lysosomal enzymes
being involved in guinea pig macrophage antimycobacterial activity. It was reported that the activity of the lysosomal enzyme, N-acetyl-β-glucosaminidase, increased in guinea pig alveolar macrophages on infection with *M. tuberculosis* H37Rv (Chandrasekhar and Mukherjee, 1990). However, β-galactosidase activity remained stable. In addition, it was found that the greater the lysosomal enzyme content of the alveolar macrophage, the fewer the intracellular acid-fast bacilli (Chandrasekhar and Mukherjee, 1990).

Lysosomal enzymes have been implicated in human macrophage tuberculocidal activity (Jaswal et al, 1993). The activities of β-glucuronidase and N-acetyl-β-glucosaminidase were observed to be significantly higher in the blood monocytes of untreated pulmonary tuberculosis patients compared to healthy individuals. After antituberculosis therapy was completed, β-glucuronidase and N-acetyl-β-glucosaminidase activities declined and their levels became comparable to those in control subjects. Although it is always possible that the increase in lysosomal enzymes could have been due simply to monocyte activation (section 1.7.), the results obtained with guinea pig macrophages (Table 3.9.) indicate that the lysosomal enzymes of human macrophages have a part to play in antimycobacterial activity.

There is mounting evidence that the contents of the lysosome are important in the antimycobacterial defence of macrophages. The nature of the tuberculocidal factors present in the lysosomes of activated guinea pig alveolar macrophages should now be determined. In addition, it needs to be established whether *M. tuberculosis* is exposed to the lysosomal contents of activated guinea pig macrophages or whether phagosome-lysosome fusion can be inhibited by the mycobacterium. To this end, attempts should be made to repeat the experiments of Armstrong and D'Arcy Hart (1971 and 1975), Clemens and Horwitz (1995) or McDonough et al (1993) using alveolar macrophages collected from vaccinated and non-vaccinated guinea pigs.

3.3. The Mycobactericidal Activity of Reactive Nitrogen Intermediates (RNI) and the Generation of RNI by Macrophages.

RNI, such as nitric oxide, nitrous acid, peroxynitrite and nitrogen dioxide, have been shown to be toxic to a large number of microorganisms *in vitro* (Castellani and Niven, 1955;

It is not certain if human macrophages are capable of generating RNI when infected. There are a number of conflicting reports in the literature (for example see Munoz-Fernandez et al, 1992b; Murray and Teitelbaum, 1992; Barnewall and Rikihisa, 1994). As human monocytes and macrophages cannot be reproducibly activated to kill M. tuberculosis in vitro (section 3.1.), the role RNI play in the tuberculocidal activity of these cells cannot be determined. It was decided in this study to investigate the production of RNI by guinea pig macrophages in response to mycobacterial infection. By using this animal model, it was possible to take advantage of tubercle strains which exhibit a spectrum of virulence in the guinea pig (section 2.5.) and the vaccination protocol that leads to the activation of guinea pig macrophages for tuberculocidal activity in vitro (O'Brien and Andrew, 1991; O'Brien et al, 1991).

3.3.1. The Mycobactericidal Activity of RNI.

The toxicity of RNI to mycobacteria in vitro was first ascertained. The eight strains of M. tuberculosis plus M. bovis 81470 were used (section 2.5.). The aim was to gain an indication of whether RNI were important in the antimycobacterial defence of guinea pigs by relating the in vitro susceptibility of each strain to its in vivo virulence (RIV). Thus, if guinea pigs produce RNI on infection with mycobacteria it would be expected that the highly virulent strains would be more resistant to RNI in vitro than the less virulent strains. However, it must be born in mind that the RIV of each mycobacterial strain was determined by infecting non-vaccinated guinea pigs (Mitchison et al, 1963; Jckett et al, 1978). It cannot therefore be guaranteed that any results obtained from relating the in vitro susceptibility to the in vivo virulence apply to BCG-vaccinated guinea pigs.
Nitric oxide is generated from acidified, aqueous nitrite solutions via the formation and subsequent decomposition of nitrous acid (Jolly, 1964). Mycobacteria were therefore tested for susceptibility to nitrite over twenty-four hours at both neutral and acidic pH. A number of preliminary experiments were performed by J. Carmichael (Department of Microbiology and Immunology, University of Leicester) and have been reported before (Carmichael, 1992). They are included here for completeness. The nitrite concentrations that were employed in these experiments covered the range of nitrite that had been previously used to inhibit the incorporation of $[^3H]$ thymidine by M. bovis BCG at pH 6 (Flesch and Kaufmann, 1991). The results are shown in Tables 3.10. and 3.11. With the exception of 79500, the strains showed no loss of viability when incubated in the presence of sodium nitrite at pH 7 (Table 3.10.). At the highest nitrite concentration, strain 79500 significantly (P< 0.01) decreased in viability by an average of 33.33%. However, the general conclusion was made that nitrite, at pH 7, was not toxic to mycobacteria.

In contrast, the tubercle strains were sensitive to nitrite at pH 5 (Table 3.11.). The strains of M. tuberculosis showed susceptibility to nitrite in a dose-dependent manner but the susceptibility of each strain varied. For example, at 125μg ml$^{-1}$ sodium nitrite, strain H37RaHR exhibited a mean decrease in viability of 80.33% whereas strain 79500 decreased in viability by an average of 55.33%. Mycobacterium bovis was resistant to all the nitrite concentrations that were tested at pH 5. Thus, the concentration of nitrite at which the killing became statistically significant varied according to the strain. For example, the killing of M. tuberculosis H37Ra was significant (P< 0.05) at 50μg nitrite ml$^{-1}$. However, significant (P< 0.05) killing of M. tuberculosis H37Rv was not observed until 125μg nitrite ml$^{-1}$ were used.

A graph was drawn of the percentage survival of the mycobacterial strains, after a twenty-four hour exposure to 125μg ml$^{-1}$ sodium nitrite at pH 5 (Table 3.11.), against in vivo virulence (Fig. 3.3.). It was found that there was a significant positive correlation (P< 0.05; regression coefficient, 0.633) between resistance to nitrite in vitro and virulence in the guinea pig. No correlation was seen when survival data at nitrite concentrations above and below 125μg ml$^{-1}$ were analysed. At 250μg ml$^{-1}$, the concentration of nitrite was high enough to overcome the
### Table 3.10. The Effect of Nitrite on the Viability of Mycobacteria Incubated in Medium at pH7.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIV&lt;sup&gt;a&lt;/sup&gt;</th>
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<th>125 ±</th>
<th>50 ±</th>
<th>5 ±</th>
<th>0 ±</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37RaHR</td>
<td>0.11</td>
<td>96.00</td>
<td>104.67</td>
<td>97.00</td>
<td>105.67</td>
<td>109.67</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6.68</td>
<td>5.25</td>
<td>16.27</td>
<td>2.36</td>
<td>8.81</td>
</tr>
<tr>
<td>H37Ra</td>
<td>0.52</td>
<td>143.33</td>
<td>170.00</td>
<td>148.67</td>
<td>202.00</td>
<td>153.50</td>
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<td>4.99</td>
<td>4.55</td>
<td>25.50</td>
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<tr>
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<td>152.33</td>
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<td>136.00</td>
</tr>
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<td>49.40</td>
<td>33.95</td>
</tr>
<tr>
<td>79112</td>
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<td>120.67</td>
<td>124.33</td>
<td>95.33</td>
<td>103.00</td>
</tr>
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<td>13.60</td>
<td>15.52</td>
<td>2.45</td>
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<td>109.00</td>
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<td>115.33</td>
<td>102.00</td>
<td>123.00</td>
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<td>103.00</td>
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<td>103.33</td>
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<td>2.87</td>
</tr>
</tbody>
</table>

Certain experiments were performed by J. Carmichael (1992). Each value represents the mean ± S.D. of three experiments.

<sup>a</sup>Strains showing a root-index of virulence (RIV) in the guinea pig of ≥ or ≤ 1.0 were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs *in vivo* (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison *et al*, 1963; Jackett *et al*, 1978).
Table 3.11. The Effect of Nitrite on the Viability of Mycobacteria Incubated in Medium at pH5.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIV\textsuperscript{a}</th>
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<th>50</th>
<th>5</th>
<th>0</th>
</tr>
</thead>
<tbody>
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<td>H37RaHR</td>
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<td>0.00 ±</td>
<td>19.67 ±</td>
<td>37.50 ±</td>
<td>70.33 ±</td>
<td>103.00 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.00</td>
<td>9.43</td>
<td>5.50</td>
<td>11.90</td>
<td>4.32</td>
</tr>
<tr>
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<td>0.52</td>
<td>4.67 ±</td>
<td>49.33 ±</td>
<td>73.50 ±</td>
<td>100.00 ±</td>
<td>107.67 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.47</td>
<td>4.03</td>
<td>1.50</td>
<td>1.41</td>
<td>0.47</td>
</tr>
<tr>
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<td>13.00 ±</td>
<td>41.67 ±</td>
<td>81.00 ±</td>
<td>92.00 ±</td>
<td>105.33 ±</td>
</tr>
<tr>
<td></td>
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<td>11.05</td>
<td>17.63</td>
<td>32.12</td>
<td>5.00</td>
<td>32.29</td>
</tr>
<tr>
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<td>40.50 ±</td>
<td>65.33 ±</td>
<td>101.67 ±</td>
<td>95.33 ±</td>
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<td>65.67 ±</td>
<td>128.33 ±</td>
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<td>5.44</td>
<td>6.65</td>
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<td>H37Rv</td>
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<td>124.00 ±</td>
<td>124.33 ±</td>
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<td></td>
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<td>10.71</td>
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<td>I2646</td>
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<td>3.67 ±</td>
<td>29.00 ±</td>
<td>54.00 ±</td>
<td>97.00 ±</td>
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<td></td>
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<td>6.38</td>
<td>8.98</td>
<td>3.27</td>
<td>2.05</td>
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<td>1.29</td>
<td>53.33 ±</td>
<td>66.00 ±</td>
<td>67.67 ±</td>
<td>127.33 ±</td>
<td>102.00 ±</td>
</tr>
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<td></td>
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<td>5.10</td>
<td>1.25</td>
<td>23.16</td>
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<td>bovis</td>
<td>&gt;1.29</td>
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<td>100.33 ±</td>
<td>104.00 ±</td>
<td>108.33 ±</td>
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<td>14.64</td>
<td>9.93</td>
<td>7.32</td>
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</tbody>
</table>

Certain experiments were performed by J. Carmichael (1992). Each value represents the mean ± S.D. of three experiments.

\textsuperscript{a}Strains showing a root-index of virulence (RIV) in the guinea pig of \(\geq 1.0\) or \(\leq 1.0\) were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs \textit{in vivo} (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison et al, 1963; Jackett et al, 1978).
Fig. 3.3. The Relationship between Survival of Mycobacteria on Exposure to Nitric Oxide and Virulence in the Guinea Pig.

Mycobacteria were incubated in medium at pH5 in the presence of 125μg ml⁻¹ sodium nitrite for 24 hours.
resistance of the majority of tubercle strains. At 50μg ml⁻¹, differences in susceptibility were not manifested, presumably because the concentration of nitrite was sufficiently low for susceptible strains to survive.

Strains of *M. tuberculosis* were therefore susceptible, *in vitro*, to acidified nitrite. Similarly, Chan *et al* (1992) have shown that the Erdman strain of *M. tuberculosis* was killed by sodium nitrite solutions at pH4.5 but not at pH7. The reason for the increased susceptibility of less virulent tubercle strains to RNI is not known. It is possible that the target molecules are more sensitive or are at a more critical threshold of availability. Alternatively, avirulent strains may possess less efficient detoxification mechanisms. Variations between mycobacterial strains in their susceptibility to RNI have also been demonstrated with organisms that belong to the MAIS complex (section 1.3.) (Doi *et al*, 1993). It was found that sodium nitrite solutions at pH7 had no antimycobacterial activity against *M. avium* Mino, *M. intracellulare* 31F093T and *M. intracellulare* KUMS 9007 (Doi *et al*, 1993). However, at pH5, the viability of the strains decreased as the concentration of nitrite increased. *Mycobacterium avium* Mino was more susceptible to the acidified nitrite than the two *M. intracellulare* strains. *Mycobacterium intracellulare* KUMS 9007 was more sensitive than *M. intracellulare* 31F093T (Doi *et al*, 1993).

It is interesting to note that *M. intracellulare* 31F093T is a murine pathogenic strain while *M. intracellulare* KUMS 9007 is a human clinical isolate (Doi *et al*, 1993). Murine macrophages are known to generate RNI for antimycobacterial activity (Adams *et al*, 1991; Denis, 1991a; Flesch and Kaufmann, 1991; Chan *et al*, 1992). Thus, there is a selection pressure on murine mycobacterial strains to become more resistant to the toxic effects of RNI. It is not clear if human macrophages can produce RNI when infected (section 1.9.2.). However, the increased susceptibility of *M. intracellulare* KUMS 9007 to acidified nitrite, as compared to *M. intracellulare* 31F093T, could be cited as evidence for human macrophages not generating RNI as an antimycobacterial mechanism.

In acidified nitrite solutions, it can be assumed that nitric oxide is the toxic moiety. Indeed, it has been demonstrated that the inhibition of *Cryptococcus neoformans* by acidified sodium nitrite solutions was due to a low molecular weight, dissolved gas; i.e. nitric oxide

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(Alspaugh and Granger, 1991). The identity of the target for nitric oxide in mycobacteria is not known. Nitric oxide causes its toxic effects in tumour cells by binding to catalytically active iron-sulphur centres, thereby inactivating enzymes involved in respiration, energy production and cell multiplication (Hibbs et al, 1984; Lancaster and Hibbs, 1990; Pellat et al, 1990). It is believed that nitric oxide acts similarly in bacteria (Reddy et al, 1983; Stuehr et al, 1989). As an iron-containing enzyme, catalase is a potential target for nitric oxide in certain strains of M. tuberculosis (Table 2.1.). However, catalase cannot be the sole target for nitric oxide in mycobacteria because the catalase-negative strains, H37RaHR and B14S3, were still highly susceptible to the toxic action of acidified nitrite.

Iron supplements have been shown to protect eukaryotic microorganisms from the toxic action of nitric oxide. Excess iron was able to reverse the trypanostatic effect of nitric oxide gas (Vincendeau and Daulouede, 1991; Vincendeau et al, 1992) and the leishmanicidal activity of RNI-producing murine macrophages (Mauel et al, 1991). Haemoglobin inhibited the cytostatic effect mediated by nitric oxide on Cryptococcus neoformans (Alspaugh and Granger, 1991). However, it has been found that activated murine macrophages can strongly inhibit the replication of both M. tuberculosis H37Rv and M. bovis BCG in the presence or absence of ferric ammonium citrate (Rook et al, 1986a). Assuming that RNI were solely responsible for the antitubercular effect, it can be concluded that additional mycobacterial enzymes, other than iron-sulphur-containing ones, were being inactivated.

Iron was tested for its ability to protect strains of M. tuberculosis from the toxic effect of 12.5μg ml⁻¹ sodium nitrite, pH5. Both iron in the form of ferric ammonium citrate (Table 3.12.) and the iron present in catalase (Table 3.13.) were assessed. Catalase was used because it may be a target for nitric oxide in catalase-positive strains of M. tuberculosis (Table 2.1.). It was found that the maximum amount of ferric ammonium citrate and catalase that could be added to the medium was 200μg ml⁻¹ and 500U ml⁻¹ respectively. Above these values and the viability of the mycobacteria decreased. It was not known why.

A supplement of iron did increase the survival of the tubercle strains (Tables 3.12. and 3.13.). As the concentration of iron increased so did the survival of each strain. For all the strains, a minimum of 100μg ml⁻¹ ferric ammonium citrate was required before viability...
Table 3.12. The Effect of Supplementing Iron on the Viability of Mycobacteria Incubated in 125μg ml⁻¹ Sodium Nitrite, pH 5.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIV</th>
<th>% survival over 24 hrs at indicated ferric ammonium citrate concentration (μg ml⁻¹)</th>
</tr>
</thead>
<tbody>
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<tr>
<td>H37RaHR</td>
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<td>35.25 ± 31.50 ± 28.50 ± 25.50 ± 24.50 ± 3.20 1.73 1.29 2.08 1.00</td>
</tr>
<tr>
<td>H37Ra</td>
<td>0.52</td>
<td>67.25 ± 61.25 ± 55.75 ± 48.50 ± 45.25 ± 4.27 3.50 2.22 3.00 3.20</td>
</tr>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>55.75 ± 51.25 ± 47.25 ± 42.50 ± 41.00 ± 5.19 5.85 5.56 5.20 5.23</td>
</tr>
<tr>
<td>79112</td>
<td>0.92</td>
<td>64.00 ± 61.75 ± 51.75 ± 44.50 ± 38.50 ± 2.94 2.99 4.11 2.52 5.45</td>
</tr>
<tr>
<td>79500</td>
<td>0.98</td>
<td>72.25 ± 70.00 ± 66.50 ± 54.50 ± 49.25 ± 1.50 1.41 2.08 3.42 2.22</td>
</tr>
<tr>
<td>12646</td>
<td>1.17</td>
<td>39.75 ± 38.50 ± 36.75 ± 30.75 ± 27.50 ± 11.47 11.24 12.15 4.57 1.73</td>
</tr>
<tr>
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<td>78.00 ± 76.50 ± 71.75 ± 69.75 ± 64.75 ± 3.16 4.12 3.20 2.06 1.50</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of four experiments.

*Strains showing a root-index of virulence (RIV) in the guinea pig of ≥ 1.0 were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs in vivo (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison et al, 1963; Jackett et al, 1978).
Table 3.13. The Effect of Supplementing Catalase on the Viability of Mycobacteria Incubated in 125μg ml⁻¹ Sodium Nitrite, pH5.

% survival over 24 hrs at indicated catalase concentration (U ml⁻¹)

<table>
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</tr>
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<td>H37Ra</td>
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<td>3.65</td>
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</tr>
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<tr>
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<td>9.07</td>
<td>6.95</td>
<td>2.87</td>
</tr>
<tr>
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<td>50.75 ±</td>
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</tr>
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</tr>
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</tr>
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<td>48.50 ±</td>
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<tr>
<td></td>
<td></td>
<td>5.07</td>
<td>8.62</td>
<td>4.69</td>
<td>2.38</td>
</tr>
<tr>
<td>79499</td>
<td>1.29</td>
<td>102.50 ±</td>
<td>91.50 ±</td>
<td>70.25 ±</td>
<td>63.75 ±</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.32</td>
<td>4.51</td>
<td>3.86</td>
<td>4.03</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of four experiments.

aStrains showing a root-index of virulence (RIV) in the guinea pig of ≥ or ≤ 1.0 were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs in vivo (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison et al, 1963; Jackett et al, 1978).
significantly (P< 0.02) increased. A significant (P< 0.05) increase in viability of all the strains was achieved at a concentration of 50U ml⁻¹ catalase. However, it is not certain in these experiments whether the excess iron inhibited the toxicity of nitric oxide by restoring the activity of mycobacterial iron-requiring enzymes or whether the iron scavenged the nitric oxide into inactive iron-nitrosyl complexes before it came into contact with the mycobacteria.

The ability of catalase to protect mycobacteria from the toxic effects of acidified nitrite was tested with three additional strains of *M. tuberculosis* which were generously supplied by Dr Y. Zhang (St Mary's Hospital Medical School, London) (Table 3.14.). The catalase-negative *M. tuberculosis* strain 24 had been converted to catalase-positive by transforming with the *M. tuberculosis katG* gene via a plasmid (Zhang et al, 1993). Plasmid pYZ66 contained the *katG* gene and had been constructed from plasmid p18R1 (Zhang et al, 1993). *Mycobacterium tuberculosis* strains 24 and 24(p16R1) were therefore catalase-negative whereas strain 24(pYZ66) was catalase-positive. By testing the susceptibility of these three mycobacterial strains to nitrite at pH5, it was possible to ascertain if mycobacterial expression of catalase provided protection from nitric oxide.

As can be seen from Table 3.14., all three strains showed susceptibility to acidified nitrite in a dose-dependent fashion. However, strain 24(pYZ66) was significantly (P< 0.001) more resistant to the higher nitrite concentrations than strains 24 and 24(p16R1). Thus, being catalase-positive conferred additional resistance to nitric oxide. This increased resistance to nitric oxide was due solely to the presence of catalase and not to the presence of the plasmid as strain 24(p16R1) was just as susceptible to nitrite at pH5 as strain 24 (Table 3.14.).

The data presented in Table 3.14. provides evidence that the expression of catalase protects mycobacteria in the presence of acidified nitrite. A similar protective effect was seen when a range of *M. tuberculosis* strains was tested for susceptibility to sodium nitrite at pH5 (Table 3.11.); in general, the catalase-positive strains survived better than the catalase-negative strains, H37RaHR and B1453. Thus, catalase can be considered to act as a scavenger of nitric oxide, preventing the nitric oxide from inactivating other mycobacterial enzymes. This scavenging effect was seen when catalase was added as a supplement to mycobacteria incubated in acidified nitrite (Table 3.13.). The survival of strains H37RaHR and B1453 increased in the
Table 3.14. The Effect of Nitrite on the Viability of *M. tuberculosis* strain 24, strain 24(p16R1) and strain 24(pYZ66) Incubated in Medium at pH5.

<table>
<thead>
<tr>
<th>NaNO₂ concentration (μg ml⁻¹)</th>
<th>% survival over 24 hours</th>
<th>strain 24 (catalase-negative)</th>
<th>strain 24(p16R1) (catalase-negative)</th>
<th>strain 24(pYZ66) (catalase-positive)</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td></td>
<td>27.94 ± 6.33</td>
<td>22.23 ± 3.98</td>
<td>57.52 ± 4.72</td>
</tr>
<tr>
<td>125</td>
<td></td>
<td>79.30 ± 5.32</td>
<td>72.65 ± 5.68</td>
<td>101.01 ± 4.69</td>
</tr>
<tr>
<td>50</td>
<td></td>
<td>84.47 ± 3.50</td>
<td>78.27 ± 3.97</td>
<td>111.52 ± 3.06</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>103.97 ± 9.82</td>
<td>112.47 ± 4.36</td>
<td>106.87 ± 7.56</td>
</tr>
<tr>
<td>0</td>
<td></td>
<td>102.82 ± 6.55</td>
<td>105.70 ± 1.27</td>
<td>105.32 ± 6.27</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three experiments.
presence of catalase.

b. SIN-1.

SIN-1 is a compound that simultaneously releases nitric oxide and superoxide in aqueous solution (Hogg et al., 1992). Nitric oxide and superoxide can combine to form peroxynitrite which will decompose when protonated (half-life approximately one second) into hydroxyl radicals and nitrogen dioxide (Hogg et al., 1992). SIN-1 can therefore generate a number of reactive oxygen intermediates (section 1.9.1.) and reactive nitrogen intermediates (RNI).

Peroxynitrite and nitrogen dioxide are strong oxidants which cause lipid peroxidation (Sagai et al., 1984; Radi et al., 1991), sulphhydryl group oxidation (Pyor et al., 1982; Radi et al., 1991) and deoxyribose oxidation (Beckman et al., 1990). It has been proposed that peroxynitrite is the major RNI responsible for the antimicrobial activity of macrophages (Ischiropoulos et al., 1992). Peroxynitrite formation has been detected in rat alveolar macrophages activated with phorbol myristate acetate (Ischiropoulos et al., 1992). However, the production of superoxide and nitric oxide in macrophages activated with cytokines and/or microbial products was found to occur separately. Superoxide was released within minutes of the addition of the stimulus whereas nitric oxide production was found to peak twenty-four hours later (Assreuy et al., 1994; Modolell et al., 1994). Macrophages could still generate peroxynitrite by utilising the superoxide that is formed from aerobic cellular metabolism. However, it was demonstrated that the leishmanicidal activity of SIN-1 was not due to peroxynitrite because authentic peroxynitrite failed to induce any toxic effect (Assreuy et al., 1994), thus casting doubt on the hypothesis that peroxynitrite is the principal RNI responsible for macrophage antimicrobial activity.

The effect of SIN-1 on the in vitro viability of the tubercle strains was tested (Table 3.15.). All the mycobacteria were susceptible to SIN-1, with the strains showing susceptibility in a dose-dependent fashion. Each strain varied in its sensitivity to SIN-1. Thus, at 1000µg ml⁻¹ SIN-1, strain B1453 exhibited a 64.48% mean decrease in survival whereas strain 79499 decreased in viability by an average of 29.35%. The concentration of SIN-1 at which killing became statistically significant varied according to the strain. For example, the killing of H37Ra was significant (P< 0.001) at 200µg ml⁻¹ whereas the killing of 79500 was significant (P<
Table 3.15. The Effect of SIN-1 on the Viability of Mycobacteria.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIVa</th>
<th>2500</th>
<th>1000</th>
<th>500</th>
<th>200</th>
<th>100</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37RaHR</td>
<td>0.11</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td>9.24 ± 0.11</td>
<td>63.13 ± 1.53</td>
<td>109.86 ± 1.05</td>
<td>104.06 ± 0.40</td>
</tr>
<tr>
<td>H37Ra</td>
<td>0.52</td>
<td>0 ± 0</td>
<td>1.32 ± 0.42</td>
<td>20.66 ± 5.46</td>
<td>55.15 ± 4.58</td>
<td>96.81 ± 3.16</td>
<td>105.03 ± 4.09</td>
</tr>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>0.58 ± 1.00</td>
<td>35.52 ± 5.54</td>
<td>55.32 ± 5.43</td>
<td>93.62 ± 1.70</td>
<td>98.47 ± 1.53</td>
<td>99.55 ± 2.25</td>
</tr>
<tr>
<td>79112</td>
<td>0.92</td>
<td>0 ± 0</td>
<td>52.74 ± 4.98</td>
<td>61.34 ± 3.86</td>
<td>82.36 ± 4.25</td>
<td>94.20 ± 3.34</td>
<td>103.67 ± 0.73</td>
</tr>
<tr>
<td>79500</td>
<td>0.98</td>
<td>4.40 ± 0.44</td>
<td>51.95 ± 0.68</td>
<td>76.19 ± 1.05</td>
<td>101.28 ± 1.90</td>
<td>109.62 ± 5.95</td>
<td>106.04 ± 4.60</td>
</tr>
<tr>
<td>H37Rv</td>
<td>1.01</td>
<td>41.14 ± 3.01</td>
<td>65.57 ± 2.11</td>
<td>85.30 ± 3.22</td>
<td>97.08 ± 1.11</td>
<td>103.18 ± 4.35</td>
<td>96.30 ± 0.69</td>
</tr>
<tr>
<td>I2646</td>
<td>1.17</td>
<td>30.90 ± 3.08</td>
<td>51.85 ± 2.82</td>
<td>67.14 ± 1.07</td>
<td>86.78 ± 5.35</td>
<td>90.57 ± 3.75</td>
<td>99.52 ± 7.89</td>
</tr>
<tr>
<td>79499</td>
<td>1.29</td>
<td>31.60 ± 13.83</td>
<td>70.65 ± 3.66</td>
<td>74.62 ± 2.43</td>
<td>84.00 ± 1.95</td>
<td>99.98 ± 5.17</td>
<td>97.97 ± 7.09</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three experiments.

*aStrains showing a root-index of virulence (RIV) in the guinea pig of ≥ 1.0 or ≤ 1.0 were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs in vivo (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison et al, 1963; Jackett et al, 1978).
0.001) at 500μg ml⁻¹. There was a significant positive correlation between the resistance of the strains to 500μg ml⁻¹ (P< 0.005; correlation coefficient, 0.909) and 1000μg ml⁻¹ (P< 0.001; correlation coefficient, 0.925) SIN-1 and in vivo virulence (Fig. 3.4.). However, a significant correlation was not obtained with the survival data at other concentrations of SIN-1.

These results (Table 3.15.) were not unexpected, given the previous work with nitric oxide (Table 3.11.). Indeed, the pattern of susceptibility to SIN-1 exhibited by the strains of *M. tuberculosis* (Fig. 3.4.) was similar to that shown for nitric oxide (Fig. 3.3.). Strain H37RaHR was the most sensitive to 500μg ml⁻¹ SIN-1, 1000μg ml⁻¹ SIN-1 and 125μg ml⁻¹ nitrite, pH5, whilst strains H37Rv and 79499 were the least sensitive to these concentrations of SIN-1 and acidified nitrite.

SIN-1 was employed to test whether other RNI were toxic to mycobacteria and attempts were made to ascertain which breakdown product of SIN-1 was toxic to the mycobacteria. Mannitol was employed to scavenge the hydroxyl radicals formed from the decomposition of peroxynitrite. The concentrations of mannitol that were used included those used by other investigators (Zhu et al, 1992; Denicola et al, 1993). When mannitol was included in the medium, the susceptibility of *M. tuberculosis* B1453 and 79499 to 500μg ml⁻¹ SIN-1 was unaltered (Table 3.16.). Thus, hydroxyl radicals seem not to be responsible for the mycobactericidal activity of SIN-1.

Authentic peroxynitrite has been found to be toxic to *Escherichia coli* (Zhu et al, 1992) and *Trypanosoma cruzi* (Denicola et al, 1993). It was ascertained, by using mannitol, that hydroxyl radicals were not mediating either of these toxic effects (Zhu et al, 1992; Denicola et al, 1993). It was, in fact, proposed that peroxynitrite was theoretically more likely to be toxic as its oxidation reactions occur far more rapidly than the formation of hydroxyl radicals (Zhu et al, 1992).

In order to test if superoxide was mediating the mycobactericidal effect, catalase was employed to inactivate the nitric oxide generated from SIN-1. Iron could not be used because, in the presence of ferric iron, superoxide reacts to form hydroxyl radicals (Babior et al, 1975). It was found that the survival of *M. tuberculosis* H37Ra, B1453 and 79500 significantly (P< 0.001) increased when 500U ml⁻¹ catalase were included in the medium containing 500μg ml⁻¹
Fig. 3.4. The Relationship between Survival of Mycobacteria on Exposure to SIN-1 and Virulence in the Guinea Pig.

500µg ml\(^{-1}\) SIN-1.

1000µg ml\(^{-1}\) SIN-1.

Mycobacteria were incubated in the presence of SIN-1 for 24 hours.
Table 3.16. The Effect of Supplementing Mannitol on the Viability of Mycobacteria Incubated in 500μg ml⁻¹ SIN-1.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIV</th>
<th>100</th>
<th>50</th>
<th>25</th>
<th>10</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>53.44</td>
<td>56.02</td>
<td>52.02</td>
<td>56.56</td>
<td>57.45 ± 3.83</td>
</tr>
<tr>
<td>79499</td>
<td>1.29</td>
<td>74.83</td>
<td>69.29</td>
<td>72.64</td>
<td>71.84</td>
<td>78.55 ± 3.61</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three experiments.

*Strains showing a root-index of virulence (RIV) in the guinea pig of ≥ 1.0 or ≤ 1.0 were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs in vivo (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison et al, 1963; Jactett et al, 1978).
It therefore appears that superoxide is not responsible for the toxicity of SIN-1 towards mycobacteria and that nitric oxide is an important factor in the mycobactericidal activity of SIN-1. Nitric oxide could be directly toxic or may be required for the formation of other toxic molecules such as peroxynitrite and nitrogen dioxide. However, caution should be exercised in interpreting this result as the catalase may have also protected the mycobacteria by degrading any hydrogen peroxide that formed from the spontaneous dismutation of superoxide. It was decided not to use superoxide dismutase as a scavenger in the SIN-1 experiments because this enzyme degrades superoxide to hydrogen peroxide. Nitric oxide reacts with hydrogen peroxide to form the highly cytotoxic species, singlet oxygen (Noronha-Dutra et al, 1993).

Interestingly, it has been reported recently that there is a continuous production of nitric oxide in human nasal airways (Lundberg et al, 1995). The source of the nitric oxide is the epithelium of the paranasal sinuses. Nitric oxide is present in sinus air in very high concentrations (approximately 20nmoles of nitric oxide were released per minute into one sinus) and some of the nitric oxide entered the nasal cavity (Lundberg et al, 1995). It was hypothesised that this nitric oxide might participate in the host's defence against airborne pathogens (Lundberg et al, 1995). If there is a sufficient exposure to nitric oxide during inhalation, then the correlation between mycobacterial virulence and resistance to RNI (Figs. 3.3. and 3.4.) will be important in determining which mycobacteria enter the lungs in a viable state. Thus, more virulent tubercle bacilli will be more likely to survive passage through the nasal airways.

Besides the correlation between virulence and RNI resistance that has been demonstrated here (Figs. 3.3. and 3.4.), the same group of tubercle strains show a correlation between virulence and hydrogen peroxide resistance (Goren et al, 1982). Nitrogen and oxygen radicals react with a number of molecules. It is unlikely that there would be strain to strain variation in all of these targets. Perhaps just one target is crucial in determining susceptibility to both RNI and reactive oxygen intermediates. Alternatively, it can be postulated that susceptibility to reactive nitrogen and oxygen intermediates is governed by the detoxification system rather than by the amount or quality of the target molecule. The catalase-negative strains, B1453 and H37RaHR, were found to be amongst the most susceptible to RNI (Tables 3.11. and 3.15.).
Table 3.17. The Effect of Supplementing Catalase on the Viability of Mycobacteria Incubated in 500\(\mu\)g ml\(^{-1}\) SIN-1.

<table>
<thead>
<tr>
<th>STRAIN</th>
<th>RIV(^a)</th>
<th>500 ± S.D.</th>
<th>0 ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>H37Ra</td>
<td>0.52</td>
<td>46.04 ± 1.38</td>
<td>23.77 ± 1.54</td>
</tr>
<tr>
<td>B1453</td>
<td>0.77</td>
<td>68.29 ± 6.89</td>
<td>49.58 ± 4.89</td>
</tr>
<tr>
<td>79500</td>
<td>0.98</td>
<td>94.17 ± 1.38</td>
<td>67.45 ± 5.57</td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of three experiments.

\(^a\) Strains showing a root-index of virulence (RIV) in the guinea pig of \(\geq 1\) or \(\leq 1\) were termed high- and low-virulence strains respectively. RIV is a measurement of the rate at which pathological lesions develop in the organs \textit{in vivo} (Personal communication from B.W. Allen, Hammersmith Hospital, London; Mitchison \textit{et al}, 1963; Jackett \textit{et al}, 1978).
addition, transforming a catalase-negative strain into a catalase-positive one decreases the susceptibility to nitric oxide (Table 3.14.). This indicates that catalase has a role in resistance to RNI. Catalase could contribute to RNI resistance in two ways. As described earlier in this section, the iron within catalase can interact with and thus detoxify nitric oxide. Catalase also degrades the hydrogen peroxide generated by mycobacterial cell metabolism. This would prevent it from combining with nitric oxide to produce singlet oxygen (Noronha-Dutra et al, 1993). However, catalase cannot be the total explanation. For example, strains 79112 and 79500, which are catalase-positive, were more sensitive to nitric oxide than strain B1453 (Table 3.11.). Mycobacterium bovis was extremely resistant to nitric oxide (Table 3.11.) but is only weakly catalase-positive (personal communication from B.W. Allen, Hammersmith Hospital, London). Other detoxification systems must therefore exist.

It has been demonstrated that the nitric oxide generated by murine macrophages induces the soxRS regulon of phagocytosed *Escherichia coli* (Nunoshiba et al, 1995). The soxRS system has been found to protect *E. coli* from the cytotoxicity of macrophages which are producing nitric oxide (Nunoshiba et al, 1993). The soxRS regulon includes genes for superoxide dismutase, endonuclease IV, glucose-6-phosphate dehydrogenase, fumarase C, NADPH:ferredoxin oxidoreductase and antisense RNA which blocks expression of the outer membrane porin, OmpF (Nunoshiba et al, 1995 and references therein). The induction of the SoxRS regulon could therefore protect *E. coli* from nitric oxide damage in a number of ways. The SoxR protein possesses an iron-sulphur centre (Hidalgo and Demple, 1994). SoxR may bind the nitric oxide, thereby preventing it from acting on any other targets. Superoxide dismutase would diminish the formation of peroxynitrite while endonuclease IV could repair any damage to DNA caused by nitric oxide-mediated cytosine deamination (section 1.9.2.). The repression of OmpF may help prevent the entry of nitric oxide into the cell. Acting together these multiple functions may be able to detoxify most of the nitric oxide generated from activated macrophages. Perhaps a similar system exists in mycobacteria.

3.3.2. The Generation of RNI by Macrophages.

Nitrite and nitrate are stable end products of the oxidative reactions of RNI. When
Macrophages produce RNI, nitrite and nitrate accumulate in the culture supernatant in a ratio of three to two (Stuehr and Marietta, 1985 and 1987). Nitrite can be measured spectrophotometrically by using the Greiss reagent (Green et al, 1982). The detection of nitrite in the culture medium is therefore frequently used as an indication of RNI synthesis by macrophages (for example see Stuehr and Marietta, 1987; Ding et al, 1988; Murray and Teitelbaum, 1992; Cunha et al, 1993). However, the nitrite concentration of the culture medium itself must always be taken into account to avoid erroneous results. It was found in this study that RPMI 1640 medium contained approximately 1.29nmoles ml⁻¹ nitrite.

Macrophages possess an inducible form of nitric oxide synthase (iNOS) and there is commonly a six to twelve hour lag between adding the stimuli and the production of RNI (Iyengar et al, 1987; Stuehr and Marietta, 1987). For maximum induction of RNI, a time interval of approximately twenty-four hours is required (Assreuy et al, 1994; Modolell et al, 1994). Thus, nitrite in culture supernatants is only measured after at least twenty-four hours incubation (Stuehr and Marietta, 1987).

Nitrite is found in the culture supernatants of murine macrophages infected with mycobacteria (Stuehr and Marietta, 1987; Adams et al, 1991; Denis, 1991a; Flesch and Kaufmann, 1991; Chan et al, 1992). Therefore, as a positive control for my experiments, the murine macrophage cell line, J774, was tested for RNI production over twenty-four hours (Table 3.18). Significantly (P< 0.001) greater amounts of nitrite were induced when two stimuli were included in the medium. For example, 5.16nmoles of nitrite were detected with interferon-γ plus M. tuberculosis H37Rv but only 2.42nmoles of nitrite were measured when interferon-γ was used alone. Interferon-γ in combination with LPS was the most potent stimulus, inducing the production of 19.23nmoles of nitrite by the J774 monolayer.

J774 macrophages produced significantly (P< 0.02) more nitrite when M. tuberculosis H37Ra was employed as a stimulus than when M. tuberculosis H37Rv was added (Table 3.18). Other researchers have found that the lipoarabinomannan of strain H37Ra is a stronger inducer of RNI synthesis in murine macrophages compared to the lipoarabinomannan of virulent M. tuberculosis H37Rv or M. tuberculosis Erdman (Adams et al, 1993; Roach et al, 1993; Schuller-Levis et al, 1994; Roach et al, 1995). Lipoarabinomannan from strain H37Ra, but not...
Table 3.18. Nitrite Production by the Murine Macrophage Cell Line, J774.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>+ IFN</th>
<th>- IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>7.50 ± 0.87</td>
<td>ND</td>
</tr>
<tr>
<td>H37Ra</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. tuberculosis</em></td>
<td>5.16 ± 0.37</td>
<td>ND</td>
</tr>
<tr>
<td>H37Rv</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>19.23 ± 2.12</td>
<td>ND</td>
</tr>
<tr>
<td>None</td>
<td>2.42 ± 0.64</td>
<td>0.01 ± 0.00</td>
</tr>
</tbody>
</table>

ND: Not Done

All values represent the mean ± S.D. of four replicates.

*Cells were grown in medium with or without 50U ml⁻¹ interferon-γ for 48 hours before the inclusion of a second stimulus. The concentration of nitrite in the culture supernatant was tested 24 hours after the second stimulus was added.*
strains H37Rv or Erdman, can elicit secretion of tumour necrosis factor-α from murine macrophages (Adams et al, 1993; Roach et al, 1993; Roach et al, 1995). The tumour necrosis factor-α will further stimulate inducible nitric oxide synthase (iNOS) production, leading to an even larger amount of RNI synthesis by murine macrophages infected with avirulent M. tuberculosis H37Ra.

The correlation between mycobacterial resistance to RNI in vitro and virulence in vivo (Figs. 3.3. and 3.4.) provides indirect evidence that guinea pigs produce RNI as a tuberculocidal mechanism. Thus, mycobacteria that are more virulent in the guinea pig are better at surviving the toxicity of acidified nitrite or SIN-1. In a mycobacterial infection, the most likely source of RNI production is the macrophage. Direct evidence was sought by testing guinea pig macrophages in vitro for RNI synthesis. Alveolar macrophages from vaccinated and non-vaccinated guinea pigs were tested for RNI production over forty-eight hours (Table 3.19.). Nitrite was not detected in the culture supernatant, even though a number of mycobacterial strains, which covered a range of virulence, and LPS were used as stimulants. In contrast, the macrophages were capable of releasing hydrogen peroxide on stimulation (Table 3.20.).

An alternative method for detecting the induction of RNI synthesis is to assay the activity of iNOS in cell homogenates (Salter et al, 1991; Severn et al, 1993). This technique involves measuring the conversion of radiolabelled arginine to citrulline by iNOS. Salter et al (1991) used this method to assay the activity of iNOS in guinea pig lung tissue, which is rich in alveolar macrophages. However, even when endotoxin from Salmonella typhimurium was present, iNOS activity was not detected by Salter and colleagues (1991). Thus, guinea pig alveolar macrophages do not seem to be able to synthesise RNI in vitro, at least not with the stimulants so far tested. Using the procedure of Salter et al (1991) and Severn et al (1993), it would be worthwhile to compare the activity of iNOS in macrophage homogenates from BCG-vaccinated guinea pigs and non-vaccinated guinea pigs after being stimulated with mycobacteria in vitro. These experiments would either confirm or refute the findings with guinea pig macrophages and the Greiss reagent (Table 3.19.).

It cannot be concluded that guinea pig macrophages do not produce RNI in vivo. In the majority of cases, it has been shown that human macrophages do not generate RNI in vitro (for
Table 3.19. Nitrite Production by Guinea Pig Alveolar Macrophages.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>VACCINATED GUINEA PIGS</th>
<th>NON-VACCINATED GUINEA PIGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis H37Ra</td>
<td>0 ± 0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis B1453</td>
<td>0 ± 0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>M. tuberculosis 79499</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>M. bovis</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>LPS</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>None</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
<tr>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
</tr>
</tbody>
</table>

ND: Not Done

Two guinea pigs were used for each experiment. All values represent the mean ± S.D. of triplicate monolayers.

aThe concentration of nitrite in the culture supernatant was tested 24 and 48 hours after the stimulus was added.
Table 3.20. Hydrogen Peroxide Production by Alveolar Macrophages from Vaccinated Guinea Pigs.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>nmoles HYDROGEN PEROXIDE/10^6 CELLS/HOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.50 ± 0.39</td>
</tr>
<tr>
<td>PMA</td>
<td>6.72 ± 0.51</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>4.11 ± 0.24</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of four monolayers. The monolayers were obtained from two guinea pigs.
example see Cameron et al, 1990; Murray and Teitelbaum, 1992; Barnewall and Rikihisa, 1994). Yet in the sera of septic patients (Hegesh and Shiloah, 1982; Ochoa et al, 1991) or the urine of cancer patients receiving interleukin-2 therapy (Hibbs et al, 1992; Ochoa et al, 1992) there is an increase in nitrate concentration. This indicates that RNI are being synthesised by an inducible NOS, although it is not certain that the induction of NOS is taking place in the macrophage. For example, iNOS activity has also been demonstrated in human neutrophils (Schmidt et al, 1989; Wright et al, 1989) and human hepatocytes (Nussler et al, 1992; Geller et al, 1993; Mellouk et al, 1994).

Mice that are infected with M. bovis BCG excrete a high level of nitrate during the course of the disease (Stuehr and Marletta, 1987) and oral administration of an inhibitor of iNOS blocks nitrate excretion by infected mice (Granger et al, 1991). When macrophages are harvested from the mice, they produce nitrite and nitrate in vitro (Stuehr and Marletta, 1985 and 1987). It would be useful if these experiments were repeated with guinea pigs as the results would provide an indication of iNOS activity in vivo. However, it would be difficult to establish that any increase in RNI production, in response to mycobacterial infection in vivo, was due to macrophage iNOS alone. For example, guinea pig liver tissue has been shown to possess an inducible NOS (Salter et al, 1991).

\(N^G\)-monomethyl-L-arginine (\(N^G\)MMA) is a monomethylated arginine analogue and is an irreversible inhibitor of iNOS (Marletta, 1993). When \(N^G\)MMA is added to macrophages, the concentration of nitrite in the culture medium is drastically reduced (Hibbs et al, 1987). \(N^G\)MMA significantly (P< 0.001) inhibited the production of nitrite by activated J774 cells over twenty-four hours (Table 3.21.). It has also been used extensively to inhibit the antimycobacterial activity of activated murine macrophages by preventing the production of RNI (Adams et al, 1991; Denis, 1991a; Flesch and Kaufmann, 1991; Chan et al, 1992).

Alveolar macrophages harvested from vaccinated guinea pigs have been previously shown to kill M. tuberculosis B1453 in vitro (O'Brien and Andrew, 1991; O'Brien et al, 1991). In addition, strain B1453 was highly susceptible to killing by RNI (Tables 3.11. and 3.15.). Thus, to complete this study, \(N^G\)MMA was tested for its ability to inhibit the killing of strain B1453 by macrophages collected from two vaccinated guinea pigs (Table 3.22.). If activated guinea pig
Table 3.21. The Inhibition by N\(^\text{G}\)MMA of Nitrite Production by J774 Cells.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>+ N(^\text{G})MMA</th>
<th>- N(^\text{G})MMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis H37Ra</td>
<td>0.57 ± 0.29</td>
<td>7.76 ± 0.42</td>
</tr>
<tr>
<td>LPS</td>
<td>0.86 ± 0.32</td>
<td>17.31 ± 1.20</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of four replicates.

\(^a\)Cells were grown in medium with 50U ml\(^{-1}\) interferon-\(\gamma\) for 48 hours before the inclusion of the second stimulus. Half of the monolayers also received 500\(\mu\)M N\(^\text{G}\)-monomethyl-L-arginine (N\(^\text{G}\)MMA). The concentration of nitrite in the culture supernatant was tested 24 hours after the stimulus was added.
Table 3.22. The Killing of \textit{M. tuberculosis} B1453 by Alveolar Macrophages from Two Vaccinated Guinea Pigs.

**Experiment 1.**

<table>
<thead>
<tr>
<th>TIME (HRS)</th>
<th>MEAN CFU ± S.D.</th>
<th>+ N^GOMMA</th>
<th>- N^GOMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Total</td>
<td>25555 ± 894</td>
<td>25455 ± 285</td>
</tr>
<tr>
<td></td>
<td>Monolayer associated</td>
<td>20575 ± 877</td>
<td>20600 ± 476</td>
</tr>
<tr>
<td></td>
<td>Monolayer supernatant</td>
<td>4980 ± 242</td>
<td>4855 ± 212</td>
</tr>
<tr>
<td></td>
<td>Bacteria alone</td>
<td>2700000 ± 215096</td>
<td>2680000 ± 240831</td>
</tr>
<tr>
<td>24</td>
<td>Total</td>
<td>18120 ± 2016</td>
<td>17160 ± 1418</td>
</tr>
<tr>
<td></td>
<td>Monolayer associated</td>
<td>15000 ± 1892</td>
<td>14225 ± 1578</td>
</tr>
<tr>
<td></td>
<td>Monolayer supernatant</td>
<td>3120 ± 156</td>
<td>2935 ± 235</td>
</tr>
<tr>
<td></td>
<td>Bacteria alone</td>
<td>3280000 ± 94516</td>
<td>3180000 ± 161245</td>
</tr>
</tbody>
</table>

**Experiment 2.**

<table>
<thead>
<tr>
<th>TIME (HRS)</th>
<th>MEAN CFU ± S.D.</th>
<th>+ N^GOMMA</th>
<th>- N^GOMMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Total</td>
<td>25575 ± 368</td>
<td>30175 ± 759</td>
</tr>
<tr>
<td></td>
<td>Monolayer associated</td>
<td>21200 ± 535</td>
<td>24875 ± 750</td>
</tr>
<tr>
<td></td>
<td>Monolayer supernatant</td>
<td>4375 ± 291</td>
<td>5300 ± 219</td>
</tr>
<tr>
<td></td>
<td>Bacteria alone</td>
<td>2510000 ± 140475</td>
<td>2580000 ± 123827</td>
</tr>
<tr>
<td>24</td>
<td>Total</td>
<td>17435 ± 2881</td>
<td>19670 ± 2727</td>
</tr>
<tr>
<td></td>
<td>Monolayer associated</td>
<td>14250 ± 2714</td>
<td>16675 ± 3242</td>
</tr>
<tr>
<td></td>
<td>Monolayer supernatant</td>
<td>3185 ± 399</td>
<td>2995 ± 532</td>
</tr>
<tr>
<td></td>
<td>Bacteria alone</td>
<td>2980000 ± 164519</td>
<td>3120000 ± 132162</td>
</tr>
</tbody>
</table>

The results show the total number of bacterial colony forming units (CFU) in the well, the number of monolayer-associated bacteria and the number of bacilli recovered from the monolayer supernatant. The number of mycobacteria in medium alone was also recorded. All values represent the mean ± S.D. of four replicate wells.

\textsuperscript{a}Monolayers and bacteria were incubated with 500\mu M N\textsuperscript{G}-monomethyl-L-arginine (N\textsuperscript{GOMMA}) during the infection period and for the subsequent 24 hour culture.
macrophages generate RNI for *in vitro* tuberculocidal activity, there should be an increase in the survival of *M. tuberculosis* B1453 in the presence of N^O_MMA. This experiment with N^O_MMA was undertaken even though nitrite had not been detected in the culture supernatants of guinea pig macrophages stimulated with mycobacterial strains (Table 3.19.). It was done to confirm that the set of negative results obtained with the Greiss reagent (Table 3.19.) was correct. The concentration of N^O_MMA that was employed was identical to that used by many other researchers to inhibit iNOS (for example see James and Glaven, 1989; Chan *et al*, 1992; Cillari *et al*, 1994; Goodrum *et al*, 1994).

In the absence of the inhibitor, macrophages were able to significantly (P< 0.002) kill an average of 33.70% of the mycobacteria over twenty-four hours (guinea pig one: 32.59%, guinea pig two: 34.81%) (Table 3.22.). In the presence of the inhibitor, the macrophages significantly (P< 0.01) killed a mean 30.46% of the bacteria (guinea pig one: 29.09%, guinea pig two: 31.83%) (Table 3.22.). *Mycobacterium tuberculosis* B1453 was not killed when incubated in medium alone, with or without N^O_MMA (Table 3.22.). Thus, RNI were not essential for the *in vitro* killing of *M. tuberculosis* B1453 by activated guinea pig macrophages.

The correlation between mycobacterial virulence and RNI resistance (Figs. 3.3. and 3.4.) does not appear to be due to activated guinea pig macrophages producing RNI as an antmycobacterial mechanism (Tables 3.19. and 3.22.). Perhaps the generation of RNI by cells other than macrophages is responsible for the correlation. For example, Salter *et al* (1991) have demonstrated that guinea pig liver tissue possesses inducible NOS activity. Alternatively, the correlation between mycobacterial virulence and RNI resistance may be a manifestation of some other variation between the strains. There may be mycobacterial systems that are capable of repairing nitric oxide damage but whose main function is to repair damage inflicted by other toxic agents, such as hydrogen peroxide. Strain variations in these systems would be manifested as differing sensitivities to RNI.

The human monocyte cell line, U937, was also tested for nitrite production over forty-eight hours (Table 3.23.). Cells that had been activated with interferon-γ were included. None of the ten mycobacterial strains that were employed could stimulate RNI synthesis. Similarly, LPS failed to elicit the generation of RNI by U937 cells (Table 3.23.). In contrast, treatment with
Table 3.23. Nitrite Production by the Human Monocyte Cell Line, U937.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>nmoles NITRITE/10⁵ CELLS&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+IFN</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37RaHR</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Ra</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> B1453</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79112</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79500</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> H37Rv</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> I2646</td>
<td>0</td>
</tr>
<tr>
<td><em>M. tuberculosis</em> 79499</td>
<td>0</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG</td>
<td>0</td>
</tr>
<tr>
<td><em>M. bovis</em></td>
<td>0</td>
</tr>
<tr>
<td>LPS</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
</tbody>
</table>

Each value represents the mean of at least two experiments.

<sup>a</sup>Cells were grown in medium with or without 100U ml⁻¹ interferon-γ for 48 hours before the inclusion of the second stimulus. The concentration of nitrite in the culture supernatant was tested 24 and 48 hours after the stimulus was added.
interferon-γ for forty-eight hours activated the U937s for release of hydrogen peroxide in response to both PMA and strains of *M. tuberculosis* (Table 3.24.). U937s can therefore be activated for an increased respiratory burst but not for the production of RNI.

Salvemini *et al* (1989) also found that U937 cells did not release nitric oxide, either in the presence or absence of LPS. The data obtained from these experiments with U937s (Table 3.23.; Salvemini *et al*, 1989) supports those researchers who have reported that human monocytes and monocyte-derived macrophages do not produce RNI in response to cytokines (Murray and Teitelbaum, 1992; Padgett and Pruett, 1992; Dumarey *et al*, 1994; Schneeman *et al*, 1994; Zembala *et al*, 1994) or infectious agents (Cameron *et al*, 1990; Murray and Teitelbaum, 1992; Bermudez, 1993; Barnewall and Rikihisa, 1994), including *M. tuberculosis* H37Rv (Dumarey *et al*, 1994). Further evidence for the involvement of RNI in the antimycobacterial activity of human macrophages will have to wait until a reproducible system is devised in which human macrophages are activated to kill *M. tuberculosis* in vitro. If such a system existed, the concentration of nitrite in the culture supernatant could be measured with the Greiss reagent, the activity of iNOS could be determined using radiolabelled arginine and the effect of NαMMA on the tuberculocidal activity could be assessed.

There is an increasing amount of evidence accumulating that the generation of RNI for antimicrobial activity in vitro is species-specific. It has been convincingly demonstrated that murine and rat macrophages generate RNI for antimicrobial purposes (section 1.9.2.; Chesrown *et al*, 1994; Green and Nacy, 1994). However, the importance of RNI in the antimicrobial activity of guinea pig and human macrophages still remains uncertain. The experiments undertaken in this thesis and the findings of Salter *et al* (1991) indicate that guinea pig alveolar macrophages do not generate RNI as a defence against microorganisms. As for human macrophages, some authors report a failure to induce RNI production on infection (Cameron *et al*, 1990; Murray and Teitelbaum, 1992; Barnewall and Rikihisa, 1994) whilst others find that the generation of RNI is an effective antimicrobial mechanism (Munoz-Fernandez *et al*, 1992b). Of particular note is the data that has been obtained with human macrophages infected with strains of *M. avium*. Human monocyte-derived macrophages were reported to produce RNI in response to infection by *M. avium* strains CIPT 14031096 (Dumarey *et al*, 1994) and LR/149 (Denis,
Table 3.24. Hydrogen Peroxide Production by U937 Cells.

<table>
<thead>
<tr>
<th>STIMULUS</th>
<th>nmoles HYDROGEN PEROXIDE/10^6 CELLS/HOUR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+IFN^a</td>
</tr>
<tr>
<td>None</td>
<td>1.37 ± 0.08</td>
</tr>
<tr>
<td>PMA</td>
<td>7.21 ± 0.05</td>
</tr>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>2.39 ± 0.10</td>
</tr>
<tr>
<td>M. tuberculosis H37Ra</td>
<td>2.40 ± 0.10</td>
</tr>
</tbody>
</table>

All values represent the mean ± S.D. of triplicate wells.

^aCells were grown in medium supplemented with 100U ml^-1 interferon-γ for 48 hours.

^bCells were grown in medium alone for 48 hours.
1991c) but not strain 101 (Bermudez, 1993). Variation in the ability to induce RNI generation by guinea pig macrophages (Table 3.19.) and human monocytes (Table 3.23.) was not seen with strains of *M. tuberculosis*.

It is possible that the general failure to detect iNOS activity in cultured guinea pig macrophages and cultured human monocytes is due to an inappropriate activation protocol. Alternatively, inhibitors of iNOS synthesis, such as interleukin-10 (Cunha et al, 1992; Oswald et al, 1992a) or transforming growth factor-β (Ding et al, 1990; Oswald et al, 1992a), may be produced during macrophage activation and infection, thereby preventing iNOS activity *in vitro*. Indeed, it has been reported that macrophages generate interleukin-10 (Barnes et al, 1992a) and transforming growth factor-β (Toossi et al, 1995) on exposure to mycobacterial products. Antibodies against these cytokines could be employed to determine if iNOS activity was restored. Finally, human and guinea pig macrophages may not possess a functional iNOS enzyme. This is perhaps due to a lack of cofactors, although it has been shown that the addition of the cofactor, tetrahydrobiopterin, to cytokine-stimulated human macrophages still does not result in any detectable accumulation of RNI (Sakai and Milstein, 1993; Schneemann et al, 1993; Michalitsyn et al, 1995).

3.4. The Activity of Ornithine Decarboxylase (ODC) in Macrophages.

The enzyme, ODC, is involved in cellular activation (Heby, 1981; Kaczmarek and Kaminska, 1989; Kaczmarek et al, 1992). Expression of ODC stimulates cell proliferation and differentiation via the formation of the polyamines, spermine and spermidine (Fig. 1.1.; Heby, 1981; Kaczmarek and Kaminska, 1989). ODC production can be induced *in vitro* by the addition of cytokines, hormones or bacterial products to cells (Bachrach, 1980; Nichols and Prosser, 1980; Messina et al, 1990; Kaczmarek et al, 1992).

ODC is also the first enzyme in a biochemical pathway that eventually leads to the formation of aminoaldehydes (Figs. 1.1. and 1.2.; Bachrach and Persky, 1964; Morgan, 1985; Levitz et al, 1990). Spermine and spermidine are deaminated by polyamine oxidase (PAO) to form aminoaldehydes. There are two types of PAO. One is designated Bovine Plasma PAO because it...
is present in the sera of ruminants. The other is called Tissue PAO/Retroplacental Serum PAO as it is found in a wide range of mammalian tissues, including macrophages, and in the sera of pregnant women (Morgan, 1980 and 1985). Bacteriophages, bacteria, fungi, protozoa, helminths, nematodes and eukaryotic cells have all been shown to be sensitive to the toxic effect of aminoaldehydes (Bachrach et al., 1963; Bachrach and Persky, 1964; Rzepczyk et al., 1984; Morgan, 1985; Ferrante et al., 1986; Levitz et al., 1990). In particular, the aminoaldehydes have been demonstrated to be toxic to *M. tuberculosis* in vitro (Bachrach and Persky, 1964). It can therefore be postulated that activated macrophages may kill tubercle bacilli by generating these aminoaldehydes. If this is true, it can be hypothesised that the macrophage may increase the production of aminoaldehydes for tuberculocidal activity by up-regulating the expression of ODC.

The ODC content of macrophages from vaccinated and non-vaccinated guinea pigs (O'Brien and Andrew, 1991; O'Brien et al., 1991) was therefore investigated. The purpose was to ascertain if macrophages that were activated to kill *M. tuberculosis* in vitro had an increased ODC activity compared to non-activated macrophages. ODC was assayed with radiolabelled ornithine and enzymic activity was measured by trapping $^{14}$CO$_2$ evolved during the decarboxylation of the substrate. The activity of cell homogenates was then calculated from standard curves obtained with pure enzyme.

Initially, the ODC content of interferon-γ-activated U937 cells was tested and compared to the ODC content of non-activated cells (Table 3.25.). The purpose of this was to determine the number of guinea pig alveolar macrophages that would be required in subsequent assays. The activity of ODC increased with cell number. ODC activity was greatly elevated in the activated U937s compared to the non-activated cells. This was to be expected, as treating U937 cells with interferon-γ leads to the activation of the cell with consequent cell division and differentiation.

From the data obtained with U937 cells (Table 3.25.), it was decided that 2x10$^6$ guinea pig macrophages would be suitable for use in an ODC assay. There were two reasons for choosing this number of macrophages. When 2x10$^5$ cells were tested, there was an approximately eight-fold difference in ODC content between the activated and non-activated U937s. This enabled the ODC activity of activated cells to be easily distinguishable from the ODC activity of non-activated cells. 2x10$^6$ was also the largest value that still allowed replicates to be tested from the total
Table 3.25. The ODC Content of the Human Monocyte Cell Line, U937.

<table>
<thead>
<tr>
<th>CELL NUMBER</th>
<th>ACTIVATED&lt;sup&gt;a&lt;/sup&gt;</th>
<th>NON-ACTIVATED&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>2.11</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2.66</td>
<td>0</td>
</tr>
<tr>
<td>5x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>6.17</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>4.62</td>
<td>0</td>
</tr>
<tr>
<td>8x10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>12.67</td>
<td>1.89</td>
</tr>
<tr>
<td></td>
<td>12.25</td>
<td>1.34</td>
</tr>
<tr>
<td>1x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>32.14</td>
<td>4.41</td>
</tr>
<tr>
<td></td>
<td>40.89</td>
<td>3.40</td>
</tr>
<tr>
<td>2x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>56.86</td>
<td>5.99</td>
</tr>
<tr>
<td></td>
<td>43.20</td>
<td>6.17</td>
</tr>
<tr>
<td>5x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>82.22</td>
<td>10.48</td>
</tr>
<tr>
<td></td>
<td>88.40</td>
<td>10.11</td>
</tr>
</tbody>
</table>

Each experiment was performed using cells from two separate cultures.

<sup>a</sup>Cells were grown in medium supplemented with 100U ml<sup>-1</sup> interferon-γ for 48 hours.

<sup>b</sup>Cells were grown in medium alone for 48 hours.
number of macrophages that were typically harvested from each guinea pig.

On stimulation of a cell there is a prompt and massive increase in ODC activity (Slotkin and Bartolome, 1983). In general, ODC is detectable less than one hour after the administration of the stimulant, the activity peaks within three to six hours and then it declines to basal levels within twelve to twenty-four hours (Slotkin and Bartolome, 1983). For example, Kaczmarek et al (1992) found that in human monocytes and macrophages ODC mRNA levels were significantly induced two hours after stimulation with tumour necrosis factor-α but decreased thereafter. ODC activity was observed to peak four hours after the addition of LPS to U937s (Yukioka et al, 1989).

The activity of ODC in 2x10⁶ guinea pig alveolar macrophages is shown in Table 3.26. Values ranged between 20 and 30mU for cells from both vaccinated and non-vaccinated guinea pigs. A difference in ODC content between activated and non-activated macrophages was therefore not discernible. The conclusion of the BCG vaccination schedule developed by O'Brien and colleagues (O'Brien and Andrew, 1991; O'Brien et al, 1991) usually consists of a six day period between administering the intravenous challenge injection and killing the guinea pig (section 2.10.). It was hypothesized that in this length of time ODC activity might decline and it was therefore decided to test the ODC content of guinea pig alveolar macrophages twenty-four hours after the i.v. injection of M. bovis BCG. However, there was no obvious difference between the activity of ODC in macrophages harvested twenty-four hours or six days after the final injection (Table 3.26.). It can be concluded that if ODC levels were elevated by the vaccination procedure the increase was not maintained over a twenty-four hour or six day period. The vaccination schedule used to activate guinea pig macrophages to kill M. tuberculosis in vitro does not therefore lead to a sustained increase in ODC activity within macrophages.

Subsequently, alveolar macrophages were collected from vaccinated guinea pigs that had been killed six days after the i.v. injection. The cells were then tested for ODC activity after twenty-four hours in culture. During this incubation period, half of the macrophage monolayers were stimulated by infecting them with M. bovis BCG (Table 3.27.). The ODC activity of both the infected and non-infected macrophages was again recorded as being between 20 and 30mU (Table 3.27.). The ODC content of the BCG-stimulated guinea pig macrophages was comparable to the
Table 3.26. The ODC Content of Guinea Pig Alveolar Macrophages.

<table>
<thead>
<tr>
<th>mU ODC/2x10^6 CELLS/HOUR</th>
<th>VACCINATED GUINEA PIGS</th>
<th>NON-VACCINATED GUINEA PIGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>27.14 ± 1.56 (3)^a</td>
<td>30.23 ± 7.72 (4)</td>
</tr>
<tr>
<td></td>
<td>29.24 ± 12.01 (4)^a</td>
<td>20.04 ± 2.99 (3)</td>
</tr>
<tr>
<td></td>
<td>22.93 ± 6.82 (3)^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td>23.47 ± 1.99 (3)^b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28.04 ± 0.56 (4)^b</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the mean ± S.D. of 2x10^6 macrophages taken from individual guinea pigs.

The number of replicates that were tested for ODC activity are shown in parentheses.

^a Guinea pigs were killed six days after the i.v. injection of live M. bovis BCG.

^b Guinea pigs were killed 24 hours after the i.v. injection of live M. bovis BCG.
Table 3.27. The ODC Content of Alveolar Macrophages from Vaccinated Guinea Pigs on Stimulation with *M. bovis* BCG.

<table>
<thead>
<tr>
<th>GUINEA PIG</th>
<th>+BCG</th>
<th>-BCG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>24.15</td>
<td>28.51</td>
</tr>
<tr>
<td></td>
<td>27.16</td>
<td>23.21</td>
</tr>
<tr>
<td>2</td>
<td>27.23</td>
<td>20.91</td>
</tr>
<tr>
<td></td>
<td>26.45</td>
<td>21.39</td>
</tr>
<tr>
<td>3</td>
<td>22.29</td>
<td>21.14</td>
</tr>
<tr>
<td></td>
<td>24.00</td>
<td>23.27</td>
</tr>
<tr>
<td>4</td>
<td>21.78</td>
<td>21.03</td>
</tr>
<tr>
<td></td>
<td>27.31</td>
<td>25.58</td>
</tr>
</tbody>
</table>

Each value represents the ODC activity of 2x10⁶ macrophages.

*Guinea pigs were killed six days after the i.v. injection of live *M. bovis* BCG. Four monolayers, each containing 2x10⁶ macrophages, were prepared from individual guinea pigs. Two of the monolayers were infected with 5x10⁷ *M. bovis* BCG. The macrophages were incubated for 24 hours at 37°C and 5% CO₂. The ODC activity was then assayed.
activity in non-stimulated macrophages. Thus, infecting macrophages from vaccinated guinea pigs with *M. bovis* BCG does not result in an elevated level of ODC activity twenty-four hours later.

Nichols and Prosser (1980) have previously shown that the cell wall material of *M. bovis* BCG can increase ODC expression in both murine peritoneal exudate macrophages and in the murine macrophage cell line, J774. ODC activity peaked within two to four hours but returned to control levels within eight hours (Nichols and Prosser, 1980). Any future experiments with guinea pig macrophages would therefore have to involve measuring the cellular ODC content at a time earlier than twenty-four hours, perhaps between three and five hours. However, the experiments in this thesis were concerned with ascertaining if macrophages, that were activated to kill *M. tuberculosis* *in vitro*, had a sustained increase in ODC expression compared to non-activated macrophages.

ODC also plays a crucial role in the growth and multiplication of microorganisms (Tabor and Tabor, 1985). It is known that *Escherichia coli* possesses ODC (Tabor and Tabor, 1985) and there is no obvious reason to suppose that ODC is not present in mycobacteria. However, the ODC content of BCG-infected guinea pig alveolar macrophages and non-infected macrophages was similar (Table 3.27.). It therefore appears that any increase in ODC activity due to the presence of intracellular *M. bovis* BCG was not detected. It seems likely that the bacteria were not disrupted by three cycles of freeze-thawing and therefore their ODC content was not measured.

The amount of ODC in 2x10^6 guinea pig alveolar macrophages was, on average, 25.07mU. This is approximately four times larger than the ODC activity of non-activated U937s (Table 3.25.). This difference may have been due simply to the fact that the alveolar macrophage is more differentiated and more metabolically active than the U937 cell (Balaji et al, 1995). Alternatively, alveolar macrophages may be in a constant state of activation, due to the continual phagocytosis of inhaled debris, and will therefore have a permanently high level of ODC expression.

ODC is a crucial enzyme in the process of cell activation, proliferation and differentiation (Russell and Snyder, 1968). It has been demonstrated that the respiratory burst (Kaczmarek et al, 1992; Messina et al, 1992), the production of reactive nitrogen intermediates (Morgan, 1994a) and the phagocytosis of *Trypanosoma cruzi* (Kierzenbaum et al, 1987) were diminished
in human macrophages when ODC was inhibited. There are a number of complex mechanisms that regulate ODC expression. These include increased or decreased enzyme turnover, transition between an active and a less active form, translational control by polyamines and antizyme induction (McCann, 1980; Pegg et al., 1994). Such an array of regulatory mechanisms illustrates the importance of controlling ODC activity and it is therefore not surprising that ODC expression returns to a basal level soon after a stimulation event.

The production of aminoaldehydes may be a mechanism by which activated macrophages kill *M. tuberculosis*. The macrophage may increase the generation of aminoaldehydes by up-regulating the expression of ODC. If ODC activity is elevated, it is certainly not maintained over a twenty-four hour period (Tables 3.26. and 3.27.). However, it is possible that an increase in ODC activity may occur transiently, which would be similar to the phenomenon that has been observed by Nichols and Prosser (1980), and even a short-term increase in ODC activity may be sufficient to allow large amounts of aminoaldehydes to be generated for tuberculocidal activity.

Alternatively, if it is hypothesised that alveolar macrophages are in a state of permanent activation because of the constant phagocytosis of inhaled debris, a high level of aminoaldehydes may be being constantly produced. Mycobacteria may therefore be killed immediately after undergoing phagocytosis. Indeed, Crowle (1988) has reported that one half of the total number of viable tubercle bacilli phagocytosed by macrophages are killed as they are ingested. However, if this scenario is true, there should be no difference in the ability of macrophages from vaccinated and non-vaccinated guinea pigs to kill *M. tuberculosis in vitro* (O'Brien and Andrew, 1991; O'Brien et al., 1991). Perhaps macrophages from BCG-vaccinated animals possess additional or more efficient tuberculocidal mechanisms.

α-Difluoromethylornithine (DFMO) is a specific and irreversible inhibitor of ODC (Mamont *et al.*, 1978). The mechanism of action involves the decarboxylation of DFMO by ODC, followed by the addition of a metabolite to the enzyme (Mitchell *et al.*, 1992). The resultant DFMO-ODC adduct is catalytically inactive. The ability of DFMO to inhibit the tuberculocidal activity of activated guinea pig macrophages could be determined. However, exposing cells to DFMO has far reaching consequences simply because ODC is crucial in cellular activation, proliferation and differentiation (Russell and Snyder, 1968). Thus, in the presence of DFMO the
cell becomes severely functionally impaired; macrophages lose the ability to mount a respiratory burst (Kaczmarek et al., 1992; Messina et al., 1992), to generate reactive nitrogen intermediates (Morgan, 1994a) and to phagocytose pathogens (Kierzenbaum et al., 1987). Thus, if the killing of tubercle bacilli by activated macrophages was reduced in the presence of DFMO, it could be concluded that ODC has a part to play in the antimycobacterial activity of the macrophage but it would not have been clear how ODC was important. In contrast, if DFMO had no effect on the extent of tuberculocidal activity, it could be concluded that ODC is not involved in the killing of *M. tuberculosis* by activated guinea pig alveolar macrophages.

The role of aminoaldehydes in macrophage tuberculocidal activity is unclear at present. However, further research into the part aminoaldehydes have to play in the antimycobacterial activity of macrophages has been undertaken by B.J. Roberts (Department of Microbiology and Immunology, University of Leicester).

3.5. The Effect of Animal Passaging on the Susceptibility of *M. tuberculosis* H37Rv to Toxic Agents.

The technique of animal passaging involves infecting animals with mycobacteria and then recovering surviving bacilli at a later date. Whilst *in vivo*, mycobacteria that were more resistant to the immune response of the animal will have been selected. Testing the recovered population of bacteria for sensitivity to various toxic substances *in vitro* may therefore allow the identification of antimycobacterial mechanisms that are active *in vivo*.

The principal cell involved in immunity to *M. tuberculosis* is the macrophage (section 1.5.). Two potential tuberculocidal mechanisms are the generation of peroxide and nitric oxide by macrophages (section 1.9.). Hydrogen peroxide is a powerful oxidant capable of reacting with DNA (Imray and Linn, 1988) and lipids (Minotti and Aust, 1987). The bactericidal action of nitric oxide is thought to be due to the inactivation of catalytically active iron-sulphur centres (Reddy *et al.*, 1983; Stuehr *et al.*, 1989) and/or the deamination of DNA (Wink *et al.*, 1991).

A strain of *M. tuberculosis* that had been passaged once through the mouse (section 2.5.) was tested for susceptibility to hydrogen peroxide and nitric oxide *in vitro*. The passaging was
performed by Dr M.J. Colston (National Institute for Medical Research, London). For comparison, the original culture of *M. tuberculosis* H37Rv was also tested. Both the pre-passaged and post-passaged strains were supplied on solid agar. It was therefore necessary to culture the mycobacteria for seven days in 7H9 broth before the assays could be performed.

3.5.1. Toxicity of Hydrogen Peroxide.

Mycobacteria were exposed to 0.08% (v/v) hydrogen peroxide over a period of ninety minutes (Jackett *et al*, 1978; Jackett *et al*, 1981a). It has been previously shown that pH affects the toxicity of hydrogen peroxide for tubercle bacilli (Jackett *et al*, 1978; Jackett *et al*, 1981a). The pH of the medium was therefore adjusted to cover a range of values, from 4.0 to 7.5. The results were recorded as colony forming units on agar and are shown in Tables 3.28 and 3.29. Both the pre-passaged and post-passaged H37Rv survived well in the absence of hydrogen peroxide and the pH of the medium did not affect viability. These results are in agreement with those of Jackett *et al* (1981a). However, in the presence of hydrogen peroxide, the pre-passaged and post-passaged mycobacteria showed a significant (P< 0.01) decrease in viability between pH 6.5 and 7.5. Jackett *et al* (1981a) found that the survival of strain H37Rv started to decline at pH 4.5 in the presence of 0.08% (v/v) hydrogen peroxide. Bacterial viability was most affected at the higher pH values (pH7 and 7.5) (Jackett *et al*, 1981a). In actual fact, the results shown in Tables 3.28 and 3.29 are highly similar to the results Jackett and colleagues (1981a) obtained when they exposed *M. tuberculosis* H37Rv to 0.02% (v/v) hydrogen peroxide. Perhaps the strain supplied from London is more resistant to hydrogen peroxide than the H37Rv strain used by Jackett *et al* (1981a). Alternatively, it is possible that the hydrogen peroxide used in my experiments had degraded, although efforts were made to ensure that the hydrogen peroxide was fresh.

Post-passaged *M. tuberculosis* was significantly (P< 0.02) more resistant to the effects of hydrogen peroxide at pH 6.5, 7 and 7.5 compared to the pre-passaged mycobacteria. For example, at pH 7 there was a mean decrease in survival of 63.13% for the original strain and 49.09% for the passaged strain. Thus, the mycobacteria that were recovered from the mouse were more resistant to hydrogen peroxide than the original population used to infect the mouse. This may
Table 3.28. The Susceptibility of Pre-passaged *M. tuberculosis* H37Rv to Hydrogen Peroxide over a Range of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>% survival over 90 mins</th>
<th>0% H₂O₂</th>
<th>0.08% (v/v) H₂O₂</th>
</tr>
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<tbody>
<tr>
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<td>95.92 ± 4.01</td>
<td>97.68 ± 0.20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>103.15 ± 2.61</td>
<td>101.13 ± 1.66</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101.06 ± 1.52</td>
<td>97.34 ± 2.32</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>100.55 ± 6.46</td>
<td>101.21 ± 6.75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>108.87 ± 15.32</td>
<td>103.89 ± 4.17</td>
<td></td>
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<tr>
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<td>98.27 ± 6.13</td>
<td>99.84 ± 1.85</td>
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<tr>
<td>5</td>
<td>93.50 ± 4.69</td>
<td>97.47 ± 2.67</td>
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<td>101.68 ± 2.88</td>
<td>102.42 ± 1.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101.99 ± 0.72</td>
<td>98.97 ± 1.81</td>
<td></td>
</tr>
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<td>106.72 ± 5.65</td>
<td>99.69 ± 3.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>110.89 ± 7.54</td>
<td>97.44 ± 3.38</td>
<td></td>
</tr>
<tr>
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<td>96.76 ± 5.41</td>
<td>98.51 ± 1.22</td>
<td></td>
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<tr>
<td>6</td>
<td>102.20 ± 8.75</td>
<td>102.32 ± 7.35</td>
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<td></td>
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<td>102.97 ± 1.15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.55 ± 2.96</td>
<td>98.04 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>6.5</td>
<td>98.46 ± 9.81</td>
<td>44.43 ± 9.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>94.56 ± 6.21</td>
<td>38.87 ± 5.45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>101.99 ± 4.49</td>
<td>47.51 ± 0.72</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>98.46 ± 9.13</td>
<td>37.93 ± 13.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td>105.73 ± 4.29</td>
<td>30.39 ± 3.32</td>
<td></td>
</tr>
<tr>
<td></td>
<td>100.94 ± 2.10</td>
<td>42.28 ± 1.65</td>
<td></td>
</tr>
<tr>
<td>7.5</td>
<td>104.19 ± 6.75</td>
<td>26.68 ± 11.00</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.47 ± 7.74</td>
<td>23.21 ± 1.46</td>
<td></td>
</tr>
<tr>
<td></td>
<td>99.43 ± 2.62</td>
<td>30.78 ± 2.32</td>
<td></td>
</tr>
</tbody>
</table>

Three individual cultures of pre-passaged *M. tuberculosis* H37Rv were tested. Each value represents the mean ± S.D. of triplicates.
Table 3.29. The Susceptibility of Post-passaged *M. tuberculosis* H37Rv to Hydrogen Peroxide over a Range of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>0% H$_2$O$_2$</th>
<th>0.08% (v/v) H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>98.37 ± 0.63</td>
<td>97.25 ± 4.67</td>
</tr>
<tr>
<td></td>
<td>101.52 ± 10.37</td>
<td>102.56 ± 5.10</td>
</tr>
<tr>
<td></td>
<td>96.24 ± 3.93</td>
<td>110.09 ± 3.74</td>
</tr>
<tr>
<td>4.5</td>
<td>94.61 ± 5.04</td>
<td>99.90 ± 3.68</td>
</tr>
<tr>
<td></td>
<td>99.30 ± 4.55</td>
<td>103.61 ± 3.86</td>
</tr>
<tr>
<td></td>
<td>109.74 ± 12.13</td>
<td>110.78 ± 5.33</td>
</tr>
<tr>
<td>5</td>
<td>100.20 ± 5.19</td>
<td>96.34 ± 2.07</td>
</tr>
<tr>
<td></td>
<td>96.29 ± 5.74</td>
<td>107.40 ± 3.74</td>
</tr>
<tr>
<td></td>
<td>104.89 ± 12.76</td>
<td>107.66 ± 3.17</td>
</tr>
<tr>
<td>5.5</td>
<td>98.07 ± 4.06</td>
<td>99.08 ± 1.57</td>
</tr>
<tr>
<td></td>
<td>95.77 ± 4.96</td>
<td>102.56 ± 3.55</td>
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<td>98.66 ± 7.19</td>
<td>101.08 ± 3.34</td>
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<td>104.27 ± 6.77</td>
<td>88.10 ± 4.29</td>
</tr>
<tr>
<td></td>
<td>109.49 ± 4.46</td>
<td>89.76 ± 1.80</td>
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<tr>
<td></td>
<td>101.08 ± 4.68</td>
<td>103.85 ± 6.32</td>
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<td>6.5</td>
<td>105.09 ± 6.00</td>
<td>57.58 ± 5.13</td>
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<td></td>
<td>106.87 ± 3.64</td>
<td>64.28 ± 1.80</td>
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<td>108.01 ± 7.84</td>
<td>51.93 ± 6.31</td>
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<tr>
<td>7</td>
<td>103.46 ± 5.32</td>
<td>52.90 ± 3.51</td>
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<td>97.73 ± 10.53</td>
<td>52.39 ± 4.08</td>
</tr>
<tr>
<td></td>
<td>95.20 ± 5.72</td>
<td>47.43 ± 3.34</td>
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<tr>
<td>7.5</td>
<td>95.93 ± 12.10</td>
<td>42.93 ± 4.16</td>
</tr>
<tr>
<td></td>
<td>93.03 ± 4.23</td>
<td>40.76 ± 1.79</td>
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<tr>
<td></td>
<td>110.43 ± 6.26</td>
<td>29.77 ± 1.59</td>
</tr>
</tbody>
</table>

Three individual cultures of post-passaged *M. tuberculosis* H37Rv were tested. Each value represents the mean ± S.D. of triplicates.
indicate that the murine immune response to *M. tuberculosis* involves the generation of peroxide. However, it is also possible that increased resistance to peroxide may be a consequence of the mycobacteria becoming more resistant to some other factor.

A possible mechanism by which mycobacteria may become more resistant to hydrogen peroxide is an increased production of catalase. The catalase content of the pre-passaged and post-passaged H37Rv could be measured and compared to determine if catalase is contributing to the decreased susceptibility of the post-passaged strain. If post-passaged H37Rv does not have a greater amount of catalase compared to the pre-passaged bacteria, this may indicate that the increased resistance to peroxide is a consequence of the tubercle bacilli becoming more resistant to another factor.

There is a large body of published articles that conclude hydrogen peroxide is not an essential component of macrophage tuberculocidal activity (for example see Lowrie *et al.*, 1985; Fiesch and Kaufmann, 1988; O’Brien and Andrew, 1991; O’Brien *et al.*, 1991; Chan *et al.*, 1992). However, all these experiments were done with macrophages *in vitro*. Macrophages may behave quite differently *in vivo* and there has been some evidence provided by studies in guinea pigs and man that hydrogen peroxide is involved in macrophage antimycobacterial activity. Jackett *et al.* (1981a) observed that peroxide-sensitive mutants of *M. tuberculosis* were killed or inhibited in guinea pig lung and spleen to a greater extent than the peroxide-resistant parent strains. Kitahara *et al.* (1979) measured an increased production of hydrogen peroxide by monocytes from TB patients.

It must also be considered that hydrogen peroxide is being produced by a cell other than the macrophage, neutrophils being the obvious choice. Neutrophils are associated with the tuberculous granuloma (Papadimitrou and Spector, 1972; Silva *et al.*, 1989), are capable of killing *M. tuberculosis* *in vitro* (Jones *et al.*, 1970; Brown *et al.*, 1987) and can release large amounts of hydrogen peroxide in response to phagocytic stimuli (Iyer *et al.*, 1961). Perhaps the production of hydrogen peroxide by these cells selected for a population of mycobacteria more resistant to peroxide than the parent population.

Thus, the production of hydrogen peroxide has been implicated as a tuberculocidal mechanism in the immune response of mice infected with *M. tuberculosis*. However, it is not
possible to ascertain if the in vivo selection of mycobacteria more resistant to hydrogen peroxide was due to the production of peroxide by macrophages.

3.5.2. Toxicity of Nitric Oxide.

The pre- and post-passaged strains of *M. tuberculosis* H37Rv were incubated for twenty-four hours in neutral and acidified medium containing sodium nitrite. At acidic pH, sodium nitrite breaks down to form nitric oxide (Jolly, 1964). The toxicity of nitric oxide for the mycobacteria was tested by counting colony forming units on agar and the results are shown in Tables 3.30. and 3.31.

Incubating the pre-passaged and post-passaged strains in neutral medium with sodium nitrite had no effect on viability. Thus, sodium nitrite at pH7 was not toxic for *M. tuberculosis* H37Rv. These results were to be expected (section 3.3.1.). The strains also survived incubation in medium at pH5 without nitrite. Only when sodium nitrite was included in the acidic medium was viability affected, again as expected (section 3.3.1.). The survival of both pre- and post-passaged H37Rv decreased as the concentration of nitrite increased. The susceptibility of the two strains to nitric oxide was not significantly different (P> 0.05). Thus, with 250μg ml⁻¹ sodium nitrite, the viability of the pre-passaged and post-passaged strains decreased by an average of 32.38% and 34.57% respectively. The extent of the susceptibility of the pre-passaged and post-passaged tubercle bacilli to sodium nitrite at pH5 (Tables 3.30. and 3.31.) was similar to that shown by the culture of *M. tuberculosis* H37Rv that had been obtained from the Leicester University Culture collection (Table 3.11.).

Passaging *M. tuberculosis* H37Rv through the mouse does not seem to have selected for a population of mycobacteria that are more resistant to reactive nitrogen intermediates (RNI) in vitro. This is surprising because it is well documented that murine macrophages use RNI against mycobacteria, both in vitro (Adams *et al*, 1991; Denis, 1991a; Flesch and Kaufmann, 1991; Chan *et al*, 1992; Barrera *et al*, 1994) and in vivo (Chan *et al*, 1995). It is possible that a difference in susceptibility to nitric oxide does exist between the pre-passaged and post-passaged strains. The passaged mycobacteria may not be expressing in vitro the factor(s) that make them more resistant to nitric oxide in vivo. This possibility is a major drawback to the passaging
### Table 3.30. The Susceptibility of Pre-passaged *M. tuberculosis* H37Rv to Nitrite at pH7 and pH5.

<table>
<thead>
<tr>
<th>NaNO₂ conc (µg ml⁻¹)</th>
<th>pH7</th>
<th>pH5</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>102.15 ± 9.62</td>
<td>67.50 ± 9.61</td>
</tr>
<tr>
<td></td>
<td>111.63 ± 4.20</td>
<td>68.51 ± 0.83</td>
</tr>
<tr>
<td></td>
<td>103.18 ± 1.16</td>
<td>66.84 ± 1.13</td>
</tr>
<tr>
<td>125</td>
<td>117.50 ± 10.98</td>
<td>74.67 ± 3.51</td>
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<td></td>
<td>98.68 ± 1.65</td>
<td>76.98 ± 2.21</td>
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<td>98.98 ± 2.44</td>
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<td>100.38 ± 1.59</td>
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<td>107.10 ± 2.01</td>
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<tr>
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<td>101.35 ± 7.46</td>
<td>110.94 ± 2.88</td>
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</tbody>
</table>

Three individual cultures of pre-passaged *M. tuberculosis* H37Rv were tested. Each value represents the mean ± S.D. of triplicates.
<table>
<thead>
<tr>
<th>NaNO₂ conc (µg ml⁻¹)</th>
<th>pH7</th>
<th>pH5</th>
</tr>
</thead>
<tbody>
<tr>
<td>250</td>
<td>97.59 ± 4.81</td>
<td>66.38 ± 2.15</td>
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<td>93.49 ± 3.81</td>
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<td>104.45 ± 1.63</td>
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<td>125</td>
<td>102.12 ± 2.73</td>
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<td>94.61 ± 0.32</td>
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<td>107.43 ± 6.01</td>
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</tbody>
</table>

Three individual cultures of post-passaged *M. tuberculosis* H37Rv were tested. Each value represents the mean ± S.D. of triplicates.
technique as only those virulence determinants that are expressed in vitro will be detected by the toxicity tests. Unfortunately, the nature of the experimental technique means that the passaged mycobacteria have to undergo a certain amount of in vitro culture in order to isolate the bacilli from animal tissue. However, care can be taken to minimise the time spent in culture prior to testing so that the chances of detecting a virulence determinant that is only expressed in vivo are increased.

In this instance, the mycobacteria were passaged through the mouse. The guinea pig is a better animal model of human TB than the mouse (Brown, 1983; Smith and Wiegeshaus, 1989). It would therefore be desirable to passage *M. tuberculosis* through guinea pigs rather than the mouse. This experiment was not undertaken in this thesis because of a lack of containment facilities for guinea pigs. However, in the future, both vaccinated and non-vaccinated guinea pigs (section 1.8.2.) could be used to passage mycobacteria. The mechanisms utilised by activated macrophages for tuberculocidal activity could then be compared to those used by non-activated macrophages. In addition, any change to the effectiveness of an antitymbacterial mechanism, caused by the vaccination schedule, would be detected. Initially, it might be more useful to passage avirulent strains of *M. tuberculosis* (Table 2.1.) through guinea pigs. These strains are more susceptible to the tuberculocidal activity of macrophages and therefore have a greater potential for adaptation. Thus, any differences in viability between the pre- and post-passaged bacteria in the in vitro toxicity tests may be more easily discernable. Once a number of experiments had been performed with avirulent strains, virulent tubercle strains could be passaged. This could determine if guinea pigs utilise different killing mechanisms for different strains of *M. tuberculosis*.

It would be particularly interesting to test the susceptibility of pre- and post-passaged *M. tuberculosis* to the lysosomal contents of activated macrophages (section 3.2.). If passaged mycobacteria were more resistant to the contents of the lysosome than non-passaged bacteria, it could be concluded that mycobacteria are exposed to the lysosomal contents in vivo. In addition, it would be valuable to determine if tubercle bacilli passaged through guinea pigs show a greater resistance to hydrogen peroxide than non-passaged mycobacteria, as was shown with mouse-
passaged mycobacteria (Tables 3.28. and 3.29.). It would be a very controversial result if it was found that hydrogen peroxide played a part in the guinea pig immune response to \textit{M. tuberculosis} because it would contradict current opinion (section 1.9.1.).

Passaging mycobacteria through guinea pigs may provide evidence for the \textit{in vivo} production of RNI (section 3.3.2.). If the guinea pig-passaged mycobacteria had been more resistant to nitric oxide \textit{in vitro} than the non-passaged strain, it would have been a strong indication that guinea pig macrophages generate RNI when infected with \textit{M. tuberculosis} \textit{in vivo}. However, the mouse-passaged mycobacteria showed no more resistance to RNI \textit{in vitro} than the non-passaged strain, even though murine macrophages are known to produce RNI as an antmycobacterial defence mechanism. It therefore seems doubtful that the technique of passaging would provide evidence for RNI synthesis by guinea pig macrophages.

3.6. Attempts to Demonstrate an Acidification Tolerance Response (ATR) with \textit{M. smegmatis} mc2155.

The ATR is an adaptive response of \textit{Salmonella typhimurium} that protects the bacterium against extreme acidity (external pH less than 4.0) by preventing a major decrease in internal pH and thus allowing the synthesis of protective acid shock proteins (Foster, 1993). The possession of an ATR permits survival in conditions that would normally be lethal. For example, the ATR is believed to have an important role in the pathogenesis of \textit{S. typhimurium} by enabling the bacterium to survive within the macrophage phagolysosome (Foster and Hall, 1990). Demonstrating an ATR in mycobacteria would help explain the ability of \textit{M. tuberculosis} to survive within the host and cause disease. It is known that \textit{M. tuberculosis} encounters acidic conditions within the body because of the effectiveness of the antmycobacterial drug, pyrazinamide, which is only toxic at acidic pH (Konno et al, 1967; Crowle et al, 1989; Chan, 1994). Although the exact locality of acidic environments is not known, potential sites are the tuberculous lesion (Dubos, 1953) and the macrophage phagosome (Sprick, 1955; de Duve et al, 1978). Mycobacteria have been shown to inhibit the acidification of phagosomes in non-activated macrophages (Crowle et al, 1991; Sturgill-Koszycki et al, 1994) but it has not been established.
whether mycobacteria are also capable of doing this in activated macrophages.

Thus, the identification of an ATR in *M. tuberculosis* would be of considerable interest. However, any experimental investigation that involves the use of *M. tuberculosis* is hampered by the pathogenic nature and slow growth of the organism. *Mycobacterium tuberculosis* requires a high level of containment and has a generation time of fifteen to twenty hours (Smith, 1981). Two to three weeks of incubation at 37°C are required before visible colonies are formed on solid media. These facts make working with *M. tuberculosis* extremely inconvenient. Thus, it is desirable to have a faster growing, non-pathogenic model organism that can substitute for *M. tuberculosis* when designing and performing initial experiments. *Mycobacterium tuberculosis* can be used once the experimental technique is perfected.

*Mycobacterium smegmatis mc²155* (Snapper et al, 1990) was chosen as the model organism for use in experiments in this thesis to test whether mycobacteria possess an adaptive response to acid. *Mycobacterium smegmatis mc²155* has a number of advantages. The first is that it is non-pathogenic. The second is that it is a fast grower, having a doubling time of approximately two hours. The third advantage lies in the fact that genetic systems for *M. smegmatis* are better defined than for *M. tuberculosis*. If *M. smegmatis mc²155* was found to possess an ATR this would facilitate future investigations into the genetics of the response.

The ATR of *S. typhimurium* was chosen as a model to study the existence of an ATR in mycobacteria. When the experiments with *M. smegmatis* were begun, only the pre-shock stage of the log phase ATR in *S. typhimurium* had been characterised (Fig. 1.3.) (Foster and Hall, 1990). This involves the induction of the pH homeostasis system when *S. typhimurium* is exposed to a mildly acidic environment (pre-shock). The pH homeostasis system prevents a major decrease in intracellular pH when *S. typhimurium* is exposed to an extremely acidic environment (post-shock). By maintaining the intracellular pH, *S. typhimurium* is able to synthesise acid shock proteins that protect the bacterium from the acidity. The following work details efforts to ascertain whether *M. smegmatis* possessed a similar adaptive response to acid; i.e. whether exposure to mildly acidic conditions would protect *M. smegmatis* against subsequent exposure to lethal acidic conditions.
3.6.1. Eliciting an ATR with *M. smegmatis* mc²155.

In the experiments described by Foster and Hall (1990), *S. typhimurium* was exposed to an "adaptive" pH of 5.6 for one doubling (pre-shock). The pH of the culture was then decreased to a "lethal" pH of 3.3 (post-shock) and the survival of the bacteria monitored over ninety minutes. It was found that bacilli that had undergone an adaptation period survived significantly better at pH3.3 than non-adapted cultures (Foster and Hall, 1990).

This experiment (Foster and Hall, 1990) was repeated with four cultures of *M. smegmatis* (Table 3.32.). As in the report of Foster and Hall (1990), hydrochloric acid was used to alter the pH of the medium. *Mycobacterium smegmatis* was allowed to adapt at pH5.8 for one doubling (four hours). The survival of the cultures was then assessed over a four hour period once the pH of the medium had been decreased to 3.3. It was found that adapted cultures of *M. smegmatis* did not survive any better than non-adapted cultures at pH3.3 (Table 3.32.). For example, three hours after the pH was dropped to 3.3, the mean viability of adapted cultures was 74% and the mean viability of non-adapted cultures was 81%. Thus, the conditions employed by Foster and Hall (1990) to demonstrate an ATR with *S. typhimurium* failed to induce an ATR in *M. smegmatis*.

The values used by Foster and Hall (1990) for the adaptive and lethal pH were chosen for specific reasons. It had been observed that the expression of certain *S. typhimurium* genes was regulated by the pH of the medium. Maximum expression of the genes occurred at pH5.8 to 6.0 (Foster and Hall, 1990). It was also found that *S. typhimurium* tolerated exposure to pH5.8 with only a slightly slower growth rate than at pH7.4 (Foster and Hall, 1990). Therefore, Foster and Hall (1990) reasoned that, if *S. typhimurium* possessed an adaptive response to acid, induction would occur between pH5.8 and 6.0. This reasoning proved to be correct. Using a lethal pH of 3.3, there was a complete loss of viability in non-adapted cultures of *S. typhimurium* within ninety minutes but adapted cultures were approximately fifty percent viable after the ninety minutes incubation (Foster and Hall, 1990). Thus, at pH3.3, a clear difference in viability could be seen between adapted and non-adapted cultures of *S. typhimurium*. The ATR of the adapted cultures was therefore easily distinguishable.

As the pH values used by Foster and Hall (1990) did not induce an ATR in *M. smegmatis*
Table 3.32. Testing *M. smegmatis* for an ATR with Hydrochloric Acid (Adaptive pH: 5.8, Lethal pH: 3.3).

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>ADAPTED²</th>
<th>NON-ADAPTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>92</td>
<td>101</td>
</tr>
<tr>
<td>2</td>
<td>82</td>
<td>88</td>
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<tr>
<td>3</td>
<td>77</td>
<td>85</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>72</td>
</tr>
</tbody>
</table>

²All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.3 with HCl.

²The cultures were adapted at pH5.8 for 4 hours.
(Table 3.32.), attempts were made to determine more appropriate adaptive and lethal pHs for \textit{M. smegmatis}. The adaptive pH was defined as the lowest pH at which \textit{M. smegmatis} still replicated, after Foster and Hall (1990). It was assumed that if \textit{M. smegmatis} possessed an ATR, it would be most likely to be induced at this pH. The lethal pH was defined as the pH that caused extensive, but not complete, cell death.

The survival of \textit{M. smegmatis} was tested over a range of pH for four hours (Table 3.33.). The pH of the culture medium was adjusted with both hydrochloric acid and phosphoric acid. Two different acids were used in these experiments because it was hypothesised that the demonstration of an ATR in \textit{M. smegmatis} might depend on the nature of the acid. With hydrochloric acid and phosphoric acid an appropriate adaptive pH was found to be pH 5.0, as \textit{M. smegmatis} was still capable of multiplying at this pH (Table 3.33.). An appropriate lethal pH was observed to be 3.0 when using hydrochloric acid and 2.5 when using phosphoric acid (Table 3.33.). Below these pH values the cultures of \textit{M. smegmatis} were losing all viability within two hours.

The experiment of Foster and Hall (1990) was repeated using hydrochloric acid to alter the pH of the medium to an adaptive pH of 5.0 and a lethal pH of 3.0. The results are shown in Table 3.34. When considered individually, the cultures of \textit{M. smegmatis} that had undergone the adaptation period appear to survive exposure to the lethal pH better than the non-adapted cultures. For example, at the two hour time point, the viability of the four adapted cultures was 12%, 78%, 71% and 74% respectively. In contrast, the viability of the four non-adapted cultures was 7%, 63%, 59% and 60% respectively. However, when the mean viability of the adapted and non-adapted cultures was compared, only the values at the four hour time point were significantly different (P < 0.01). Thus, using an adaptive pH of 5.0 and a lethal pH of 3.0 with hydrochloric acid does not lead to a clear demonstration of an ATR in \textit{M. smegmatis}.

The experiment was also performed using an adaptive pH of 5.0, a lethal pH of 2.5 and phosphoric acid to control the pH of the medium. The results are shown in Table 3.35. The culture that had been exposed to the adaptive pH for four hours did not exhibit a greater ability to survive the lethal pH of 2.5. In fact, the viability of the adapted culture was almost identical to the viability of the non-adapted culture. Thus, \textit{M. smegmatis} did not possess an ATR under these conditions.
Table 3.33. Survival of *M. smegmatis* over a Range of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>log₁₀ CFU/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>log₁₀ CFU/ml&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>t=0</td>
<td>t=2</td>
</tr>
<tr>
<td>6.0</td>
<td>6.04</td>
<td>6.05</td>
</tr>
<tr>
<td>5.0</td>
<td>6.04</td>
<td>6.06</td>
</tr>
<tr>
<td>4.5</td>
<td>6.03</td>
<td>6.03</td>
</tr>
<tr>
<td>4.0</td>
<td>6.01</td>
<td>5.98</td>
</tr>
<tr>
<td>3.5</td>
<td>5.99</td>
<td>5.95</td>
</tr>
<tr>
<td>3.0</td>
<td>5.98</td>
<td>5.78</td>
</tr>
<tr>
<td>2.5</td>
<td>5.96</td>
<td>0.00</td>
</tr>
<tr>
<td>2.0</td>
<td>5.89</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The results show the number of bacterial colony forming units (CFU) after 0, 2 or 4 hours of incubation at each pH. All values are the mean of two counts taken from a single sample at the appropriate time point.

<sup>a</sup>HCl was used to pH the medium.

<sup>b</sup>H<sub>3</sub>PO<sub>4</sub> was used to pH the medium.
Table 3.34. Testing *M. smegmatis* for an ATR with Hydrochloric Acid (Adaptive pH: 5.0, Lethal pH: 3.0).

### % VIABILITY OF ADAPTED CULTURES

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>55</td>
<td>83</td>
<td>78</td>
<td>95</td>
<td>78 ± 17</td>
</tr>
<tr>
<td>2</td>
<td>12</td>
<td>78</td>
<td>71</td>
<td>74</td>
<td>59 ± 31</td>
</tr>
<tr>
<td>3</td>
<td>4</td>
<td>78</td>
<td>59</td>
<td>60</td>
<td>50 ± 32</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>66</td>
<td>58</td>
<td>46</td>
<td>43 ± 29</td>
</tr>
</tbody>
</table>

*aThe cultures were adapted at pH 5.0 for 4 hours. All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.0 with HCl.*

### % VIABILITY OF NON-ADAPTED CULTURES

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>34</td>
<td>69</td>
<td>68</td>
<td>93</td>
<td>66 ± 24</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>63</td>
<td>59</td>
<td>60</td>
<td>47 ± 27</td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>48</td>
<td>50</td>
<td>18</td>
<td>29 ± 24</td>
</tr>
<tr>
<td>4</td>
<td>0.3</td>
<td>14</td>
<td>15</td>
<td>2.5</td>
<td>8 ± 8</td>
</tr>
</tbody>
</table>

*bAll values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.0 with HCl.*
Table 3.35. Testing *M. smegmatis* for an ATR with Phosphoric Acid (Adaptive pH: 5.0, Lethal pH: 2.5).

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>ADAPTED(^{b})</th>
<th>NON-ADAPTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>9</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

\(^a\)All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 2.5 with H\(_3\)PO\(_4\).

\(^b\)The culture was adapted at pH5.0 for 4 hours.
A weak ATR had been observed when an adaptive pH of 5.0 and a lethal pH of 3.0 had been used with hydrochloric acid (Table 3.34.). It was therefore hypothesised that a lethal pH of 2.5 with phosphoric acid may have been too acidic to allow the demonstration of an ATR; i.e. pH2.5 is so toxic that it can overcome any protection that has been provided during adaptation. It was therefore decided to repeat the experiments with phosphoric acid using a lethal pH of 3.5. This pH kills non-adapted cultures of *M. smegmatis* over four hours but is not as toxic as lower pHs (Table 3.33.).

When the experiment was repeated with an adaptive pH of 5.0 and a lethal pH of 3.5, adapted cultures of *M. smegmatis* survived significantly ($P < 0.05$) better than non-adapted cultures at all time points (Table 3.36.). For example, after a one hour exposure to pH3.5, the mean viability of adapted cultures was $83\% \pm 14$ whilst the mean viability of non-adapted cultures was $47\% \pm 16$. Thus, *M. smegmatis* possesses a demonstrable ATR when the pH of the medium is controlled with phosphoric acid and when the mycobacterial culture is adapted at pH5.0 for one doubling before exposure to pH3.5.

When an ATR has been demonstrated in other bacteria, the difference between the survival of the adapted and non-adapted cultures has been quite substantial. The adapted bacterial cultures have been between fifty and one hundred percent more viable than the non-adapted cultures at the end of the incubation period (Goodson and Rowbury, 1989; Foster and Hall, 1990; Belli and Marquis, 1991; Kroll and Patchett, 1992; Karem *et al.*, 1994). With *M. smegmatis* the difference in viability between adapted and non-adapted cultures was not so dramatic (Table 3.36.). For the first three hours of incubation at pH3.5, adapted cultures were on average approximately thirty percent more viable than non-adapted cultures. However, after four hours exposure to pH3.5 the adapted cultures were, on average, nineteen percent more viable than the non-adapted cultures (Table 3.36.).

It is possible that *M. smegmatis* does not possess an ATR that protects against low pH as strongly as the ATR of other bacteria. *Mycobacterium smegmatis* is capable of growth over a wide pH range, from 4.6 to 7.4 (Portaels and Pattyn, 1982). Depending on the acid, *M. smegmatis* can survive four hours exposure to pH values as low as 3.0 and 2.5 (Table 3.33.). These phenomena occur in the absence of an adaptation period at a mildly acidic pH. Thus, *M. smegmatis* is naturally
Table 3.36. Testing *M. smegmatis* for an ATR with Phosphoric Acid (Adaptive pH: 5.0, Lethal pH: 3.5).

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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<td>100 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>91</td>
<td>95</td>
<td>76</td>
<td>77</td>
<td>88</td>
<td>98</td>
<td>58</td>
<td>83 ± 14</td>
</tr>
<tr>
<td>2</td>
<td>78</td>
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<td>43</td>
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<td>43</td>
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<td>36 ± 28</td>
</tr>
<tr>
<td>4</td>
<td>52</td>
<td>52</td>
<td>4</td>
<td>27</td>
<td>21</td>
<td>10</td>
<td>1</td>
<td>24 ± 21</td>
</tr>
</tbody>
</table>

*aThe cultures were adapted at pH5.0 for 4 hours. All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.5 with H$_3$PO$_4$.*

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
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<td>100</td>
<td>100</td>
<td>100</td>
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<td>100</td>
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<td>100 ± 0</td>
</tr>
<tr>
<td>1</td>
<td>57</td>
<td>69</td>
<td>60</td>
<td>35</td>
<td>28</td>
<td>50</td>
<td>32</td>
<td>47 ± 16</td>
</tr>
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<td>28</td>
<td>38</td>
<td>13</td>
<td>12</td>
<td>24</td>
<td>16</td>
<td>21 ± 10</td>
</tr>
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<td>1</td>
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<td>11</td>
<td>18</td>
<td>4</td>
<td>8 ± 6</td>
</tr>
<tr>
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<td>6</td>
<td>8</td>
<td>12</td>
<td>2</td>
<td>5 ± 4</td>
</tr>
</tbody>
</table>

*bAll values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.5 with H$_3$PO$_4$.*
resistant to acidic conditions and must therefore possess highly effective, constitutively expressed mechanisms that protect against acid. High levels of inducible acid protection are not needed in the presence of efficient constitutive systems and this may explain why a weak ATR was demonstrated with *M. smegmatis*. However, optimum conditions for the induction of the ATR in *M. smegmatis* may not have been achieved in these experiments. Perhaps an alteration in the adaptive pH or in the length of the adaptation period would have lead to a more prominent ATR. A set of experiments should be undertaken to test the effectiveness of using different adaptive pHs (values between 7.0 and 4.0) with a range of lethal pHs (values between 5.0 and 2.0). By performing these experiments, it should be possible to identify the conditions that produce a maximum ATR effect in *M. smegmatis*.

However, an alternative explanation may account for the relatively weak ATR of *M. smegmatis*. In *S. typhimurium*, the adaptive pH induces the expression of a pH homeostasis system that permits the synthesis of protective acid shock proteins at the lethal pH. This may not be the case in *M. smegmatis*. Acid shock proteins may need to be synthesised before exposure to the lethal pH; a situation that is similar to the post-shock ATR in *S. typhimurium* (Fig. 1.3.). Thus, the ATR effect with *M. smegmatis* (Table 3.36.) may be enhanced if the mycobacteria are incubated at a pH less than 5.0 for a short time after the adaptation period and before the drop to the lethal pH.

### 3.6.2. Determining the Effect of Altering the Length of the Adaptation Period on the ATR.

Based on the work of Foster and Hall (1990), cultures of *M. smegmatis* were originally exposed to the adaptive pH for four hours which was the time required for the bacteria to undergo one doubling. Experiments were subsequently performed to ascertain the minimum amount of time that was required at the adaptive pH for an ATR to be induced. Cultures of *M. smegmatis* were exposed to pH5.0 for fifteen minutes, one hour and two hours before phosphoric acid was used to decrease the pH of the medium to 3.5 (Table 3.37.). Cultures that were adapted at pH5.0 for fifteen minutes showed no more survival at the lethal pH than non-adapted cultures. However, those cultures of *M. smegmatis* that were incubated at pH5.0 for one or two hours did survive better at pH3.5 than the non-adapted cultures. Thus, *M. smegmatis* has to be exposed to the adaptive pH between fifteen minutes and one hour to allow induction of the ATR and this would
Table 3.37. The Effect of Altering the Length of the Adaptation Period on the ATR of *M. smegmatis*.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>15 mins</th>
<th>60 mins</th>
<th>120 mins</th>
</tr>
</thead>
<tbody>
<tr>
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<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
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<td>18</td>
<td>13</td>
<td>56</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>3</td>
<td>48</td>
</tr>
</tbody>
</table>

% VIABILITY OF ADAPTED CULTURES

The cultures were adapted at pH 5.0 for 15, 60 or 120 minutes. All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.5 with H$_3$PO$_4$.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>15 mins</th>
<th>60 mins</th>
<th>120 mins</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>100</td>
<td>100</td>
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<tr>
<td>1</td>
<td>48</td>
<td>43</td>
<td>98</td>
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<tr>
<td>2</td>
<td>22</td>
<td>29</td>
<td>67</td>
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<td>3</td>
<td>18</td>
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</tr>
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<td>4</td>
<td>7</td>
<td>3</td>
<td>48</td>
</tr>
</tbody>
</table>

% VIABILITY OF NON-ADAPTED CULTURES

When the culture had reached a concentration of 1x10$^8$ bacilli ml$^{-1}$, it was incubated for a further 15, 60 or 120 minutes before the pH was dropped to 3.5 with H$_3$PO$_4$. All values are the mean of two samples taken from each culture at the appropriate time point after the pH was dropped to 3.5 with H$_3$PO$_4$. 190
imply that *M. smegmatis* does not need to replicate at pH5.0 for an ATR to develop.

3.6.3. Testing the Importance of Protein Synthesis to the Development of the ATR.

The ATR of *M. smegmatis* may require the induction of genes and the synthesis of new proteins. Alternatively, the acid adaptive response may be due purely to alterations in pre-existing physiological processes, such as proton transport (section 1.11.). Experiments were performed to test if the induction of the ATR in *M. smegmatis* involved protein synthesis. An excess amount of chloramphenicol (200μg ml⁻¹) was added to cultures of *M. smegmatis* thirty minutes before the start of the adaptation period. The antibiotic was therefore present during the adaptation period and through the incubation at pH3.5, preventing the synthesis of proteins at all times during the exposure to acidity. If protein synthesis was required for the development of an ATR, the inclusion of chloramphenicol would prevent the adaptive response and render the cultures sensitive to the lethal pH. The results are shown in Table 3.38. Adapted cultures that were exposed to chloramphenicol did not survive the lethal pH as well as adapted cultures without chloramphenicol. The viability of chloramphenicol-treated cultures was similar to non-adapted cultures. The addition of chloramphenicol therefore prevented the induction of the ATR in *M. smegmatis* which indicates that protein synthesis is required for the adaptive response to acid. Similarly, protein synthesis has been shown to be necessary for the induction of an acid adaptive response in other bacteria (Foster and Hall, 1990; Kareem *et al.*, 1994).

Thus, it has been demonstrated that exposure of *M. smegmatis* to mildly acidic conditions protects against exposure to lethal acid conditions (Table 3.36.). When these experiments were originally performed, the three components of the ATR of *S. typhimurium* (Fig. 1.3.) had not been elucidated. The later experiments of Foster and colleagues (Foster, 1993; Lee *et al.*, 1994) now need to be repeated to clarify the mechanism of the ATR in *M. smegmatis*. Determining whether protein synthesis occurs at the lethal pH will distinguish between the pre-shock and post-shock response. If the adaptive response at pH5.0 is a pre-shock event, then the acid shock proteins will be synthesised at pH3.5. Alternatively, if the acid shock proteins are synthesised at the adaptive pH then the incubation at pH5.0 induces a post-shock ATR.
Table 3.38. The Effect of Chloramphenicol on the ATR of *M. smegmatis*.

<table>
<thead>
<tr>
<th>Time (hrs)</th>
<th>ADAPTED(^b)</th>
<th>ADAPTED + CHLORAMPHENICOL(^c)</th>
<th>NON-ADAPTED</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>71</td>
<td>68</td>
<td>27</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>56</td>
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<tr>
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<td>4</td>
<td>12</td>
<td>12</td>
<td>4</td>
</tr>
</tbody>
</table>

\(^a\)All values are one sample taken from each culture at the appropriate time point after the pH was dropped to 3.5 with H\(_3\)PO\(_4\).

\(^b\)The cultures were adapted at pH5.0 for 4 hours.

\(^c\)200\(\mu\)g ml\(^{-1}\) chloramphenicol were added 30 minutes before the pH was dropped to 5.0. The cultures were adapted for 4 hours.
Unfortunately, the experiments with chloramphenicol that have been described in this thesis (Table 3.38.), only indicate that protein synthesis is required at some point in the ATR. If the adaptive response of M. smegmatis (Table 3.36.) is a pre-shock event, then protein synthesis will be required both for the induction of the pH homeostasis system and subsequent production of acid shock proteins. In contrast, if the adaptive response is a post-shock event, protein synthesis will only be required for the production of protective acid shock proteins.

Research into the genetics of the ATR in M. smegmatis has been undertaken by S. Gordon (Department of Microbiology and Immunology, University of Leicester). Gene induction and protein synthesis during the adaptation phase has been studied in the hope of characterising the proteins and genes that are involved in the ATR. To date, a single promoter has been identified that is induced in response to acid stress (Gordon, 1995).

Assuming that the mycobacterial-containing phagosomes of activated macrophages become acidified, it seems doubtful that mycobacteria will be able to undergo a pre-shock type of ATR when ingested. This is because the process of phagosomal acidification will occur rapidly, thereby preventing the mycobacteria from adapting to mildly acidic conditions in preparation for the large drop in pH. It seems more likely that, if any ATR response is induced, it will be an acid shock type of response, in which acid shock proteins are synthesised upon a brief exposure to acidic pH (Fig. 1.3.). Experiments should be undertaken to determine if M. smegmatis has an acid shock response. This would involve incubating M. smegmatis in acidic medium (for example, pH 5.0 to 4.0) for a short period, perhaps thirty minutes, before decreasing the pH to a lethal value.

Although it is possible that mycobacteria could undergo an acid adaptive response during the process of phagosome acidification, there is an alternative mechanism that would allow the bacteria to survive within the macrophage phagosome. The tuberculous lesion is believed to be acidic (Dubos, 1953). The acidification of these lesions is likely to be a slow process as it is thought to be due to the anaerobic metabolism of the surrounding cells (Dubos, 1953). Under these conditions, it is probable that an ATR would develop. If the lesion liquefies, the mycobacteria will be released into the external environment and may be inhaled (section 1.4.). When the bacilli are ingested by the macrophage, they will be adapted to acidic conditions and acidification of the phagosome will therefore have no effect on them. In this way, mycobacteria may overcome
the need to develop an ATR when in the phagosome.

As it has been demonstrated that *M. smegmatis* possesses an adaptive response to acid (Table 3.36.), all these experiments need to be repeated with *M. tuberculosis*. This is because *M. smegmatis* is avirulent and cannot therefore be used to study the role of the ATR as a virulence factor. Assuming that evidence was gained for an ATR in *M. tuberculosis*, one approach that could be taken to investigate its importance in the virulence of tubercle bacilli would be to test strains of *M. tuberculosis* that cover a range of virulence (section 2.5.) for an ATR. The relationship between the strength of the adaptive response and virulence would then indicate if the ATR has a role to play in mycobacterial virulence.

*Mycobacterium tuberculosis* does not grow over a wide range of pH. It has been observed that optimal growth occurs between pH5.8 and 6.5 and that the bacteria do not multiply below pH5.4 (Portaels and Pattyn, 1982). Thus, unlike *M. smegmatis*, the constitutive mechanisms that protect the cell against acid damage do not appear to be very effective in *M. tuberculosis*. It can be hypothesised that, in the absence of high levels of constitutive acid protection, powerful inducible systems exist. It may therefore be possible to demonstrate a strong ATR effect with *M. tuberculosis*.

 Apart from increasing the survival of bacteria at extreme acidity, the induction of the ATR may protect the cell from other stresses. Although Foster and Hall (1990) originally observed that cross-protection from hydrogen peroxide exposure or heat shock did not take place when the ATR was induced in *S. typhimurium*, other researchers have reported that acid adaptation does cross-protect *S. typhimurium* from hydrogen peroxide and from heat and osmotic stress (Leyer and Johnson, 1993). Thus, assuming that the mycobacterial-containing phagosome of activated macrophages does become acidified, it can be hypothesised that, if the ATR is induced, *M. tuberculosis* is protected not only from the acidic environment but also from other toxic mechanisms of the macrophage. Indeed, below pH5.0 the susceptibility of certain strains of *M. tuberculosis* to hydrogen peroxide is enhanced (Jackett *et al*, 1978; Jackett *et al*, 1981a). It would therefore be interesting to ascertain if mycobacteria that had undergone an adaptation period were more resistant to hydrogen peroxide *in vitro* than non-adapted cultures. However, the mycobacterial promoter that was identified as being induced in response to acid stress
(Gordon, 1995) has been shown to be unresponsive to temperature or hydrogen peroxide (personal communication from S. Gordon, Department of Microbiology and Immunology, University of Leicester).

It is believed that the ATR allows the survival of *S. typhimurium* within macrophage phagolysosomes (Foster and Hall, 1990). Virulent *Salmonella* strains react to phagocytosis by a macrophage-like cell line with increased expression of at least thirty proteins (Buchmeier and Heffron, 1990) but it is not known if any of these are involved in the ATR. Plum and Clark-Curtiss (1994) have recently developed a method for identifying genes that are expressed by mycobacteria inside macrophages. If the genes responsible for the ATR in *M. tuberculosis* were characterised, they could be compared to the genes that are expressed by mycobacteria inside activated guinea pig macrophages (section 1.8.2.). This process would determine whether an ATR was induced when mycobacteria infect activated macrophages and provide evidence that mycobacteria reside inside acidic phagosomes in activated macrophages.

Alternatively, activated guinea pig macrophages (section 1.8.2.) could be infected with *M. tuberculosis* and with a mutant that lacked the ATR. The fate of the ATR-deficient strain, as compared to *M. tuberculosis*, would provide evidence for the role the ATR plays in defending mycobacteria against the antimicrobial mechanisms of the macrophage. If the ATR-deficient strain was killed to a greater extent than *M. tuberculosis*, it would indicate that the ATR was important in virulence. The ATR of *M. tuberculosis* would therefore be a drug target.
4. CONCLUSIONS.
The main aim of this thesis was to study the tuberculocidal mechanisms of activated macrophages. In order to investigate macrophage antimycobacterial activity, it is desirable to have an in vitro model that imitates in vivo conditions. In vitro models are preferred because all aspects of the interaction between the macrophage and tubercle bacillus can be controlled and manipulated by the experimenter. When studying a human disease it is always best to have a model that consists of human cells. Human macrophages are activated to kill *M. tuberculosis* in vivo by the secretion of cytokines from T-cells (section 1.5.). It should be possible to activate human macrophages for in vitro tuberculocidal activity in a similar fashion, by employing commercially-available cytokines or by generating cytokines from homologous, mycobacteria-reactive T-cells. However, most researchers have failed to activate cultured human macrophages to kill or inhibit *M. tuberculosis* (for example see Douvas *et al.*, 1985; Rook *et al.*, 1986a; Steele *et al.*, 1986; Rook, 1990). Only two reports have been published that detail procedures whereby cultured human macrophages are activated by cytokines to exert antimycobacterial activity in vitro (Crowle and May, 1981; Denis, 1991b).

Denis (1991b) was able to induce human monocytes to kill *M. tuberculosis* H37Rv by including interferon-γ, tumour necrosis factor-α and calcitriol in the culture medium. Crowle and May (1981) used supernatants from stimulated lymphocytes to activate human macrophages to inhibit the replication of *M. bovis* BCG and *M. tuberculosis* Erdman. Unfortunately, attempts to recreate these in vitro models failed (section 3.1.). The immunomodulators were not able to activate the monocytes of two donors to kill *M. tuberculosis* H37Rv over an eight day period (Tables 3.1. and 3.2.) and the lymphocyte culture supernatant did not activate the macrophages of four donors to inhibit the growth of *M. bovis* BCG over a seven day incubation period (Table 3.5.). The study in this thesis of the tuberculocidal mechanisms of human macrophages was therefore prevented. Possible reasons for the failure to repeat these experiments (Crowle and May, 1981; Denis, 1991b) and alternative experimental procedures that may allow the demonstration of human macrophage antimycobacterial activity in vitro were discussed in section 3.1.

When it is impossible to work with human cells in vitro, animal cells are the alternative. It is advantageous to choose an animal that undergoes a disease process that is similar to the human disease. In this way, any discoveries made using the animal model will be relevant to
humans. Guinea pigs are a good animal model for human tuberculosis because these animals are as susceptible to infection by *M. tuberculosis* as humans and the pathology of the disease is similar to that found in man (Brown, 1983; Smith and Wiegeshaus, 1989). It is also possible to activate guinea pig alveolar macrophages to kill *M. tuberculosis in vitro* by an *in vivo* vaccination schedule (O'Brien and Andrew, 1991; O'Brien et al, 1991).

Tuberculosis is principally a disease of the lung. Alveolar macrophages therefore have a major role to play in the defence of the host (section 1.5.). Thus, the model of O'Brien and colleagues has the additional advantage of using alveolar macrophages which are more appropriate for the study of antimycobacterial mechanisms than macrophages from other tissues. Potential tuberculocidal mechanisms of guinea pig alveolar macrophages were therefore studied in this thesis because of the absence of a reproducible human macrophage model. The contents of the lysosome (section 3.2.) and the generation of reactive nitrogen intermediates (section 3.3.) and aminoaldehydes (section 3.4.) were investigated.

It is likely that macrophages possess a number of effective antimycobacterial mechanisms and that killing of *M. tuberculosis* is not due to one single, exclusive mechanism. In actual fact, what were thought to be distinct tuberculocidal mechanisms may be strongly connected. For example, there is evidence to suggest that the ornithine required for toxic aminoaldehyde synthesis (section 1.9.2.) may be derived from arginine (Morgan, 1994b; Morgan and Baydoun, 1994). The enzyme arginase will break down arginine to form urea and ornithine. Thus, there are potentially two pathways in the cell that require arginine: the nitric oxide pathway (section 1.9.2.) and the aminoaldehyde pathway. The metabolism of arginine by the macrophage can therefore determine the response to infection.

Transforming growth factor-β has been shown to up-regulate the activity of arginase in rat peritoneal macrophages, which leads to the accumulation of polyamines (Boutard et al, 1995). This cytokine also acts to inhibit the activity of iNOS in rat peritoneal macrophages (Boutard et al, 1995). It was found that, in the absence of transforming growth factor-β, rat peritoneal macrophages were able to inhibit tumour cell proliferation. However, when the macrophages were incubated with transforming growth factor-β, the tumour continued to grow (Boutard et al, 1995). The cytostasis effect of rat peritoneal macrophages is therefore dependent
on the production of nitric oxide, which requires a low level of arginase activity. The regulation of arginase is therefore a mechanism by which the response of the macrophage to infection can be controlled.

It is interesting to note that LPS and BCG cell wall material increase both ODC activity (Nichols and Prosser, 1980) and iNOS activity (Stuehr and Marletta, 1987; Cunha et al, 1993) in murine macrophages. In these circumstances, it could be speculated that competition for arginine would arise between the two pathways. However, it has been found that maximum induction of ODC and iNOS occurs at different times. ODC activity peaks within three to six hours after the stimulation event (Slotkin and Bartolome, 1983) whereas iNOS activity is not detectable until at least six hours after the addition of the stimulus (Morgan, 1994b). Thus, it can be hypothesised that the murine macrophage may be able to mount a prolonged attack on any invading mycobacteria by generating aminoaldehydes followed by reactive nitrogen intermediates. Under these conditions, a high level of arginase activity would be required whilst ODC was active but arginase activity would have to be at a minimum during the period that iNOS was active.

However, in this study with guinea pigs, only the contents of the lysosome were definitely implicated in the tuberculocidal activity of activated macrophages (section 3.2.). The extracts from lysosomes of activated guinea pig macrophages were capable of killing significant numbers of *M. tuberculosis* H37Rv but there was no significant change in mycobacterial numbers in the lysosomal extracts of non-activated macrophages (Table 3.9.). The activity against *M. tuberculosis* was observed at pH5.5 but not pH7.0 (Table 3.9.). There was no evidence that the generation of RNI was used as an antimycobacterial mechanism by the macrophage (section 3.3.). In addition, it was found that any increase in aminoaldehyde synthesis that may take place in macrophages infected with mycobacteria was not due to elevated ODC activity twenty-four hours after a stimulation event (section 3.4.). Unfortunately, these findings with guinea pig macrophages cannot be confirmed as an accurate reflection of human macrophage tuberculocidal activity until a reproducible system is devised in which human macrophages are activated to kill *M. tuberculosis in vitro*.

It has not been resolved whether human macrophages produce RNI when infected with mycobacteria (section 1.9.2.). As human macrophages cannot be reproducibly activated to kill *M.
tuberculosis in vitro (section 3.1.), the role of RNI in the tuberculocidal activity of human macrophages is unknown. Any indication of human macrophage antimycobacterial mechanisms must come from animal models. It has been well documented that murine macrophages generate RNI as an antimycobacterial mechanism (Adams et al., 1991; Denls, 1991a; Flesch and Kaufmann, 1991; Chan et al., 1992). Using the mouse as the animal model for human TB would therefore lead to the conclusion that RNI were likely to be involved in human macrophage tuberculocidal activity. However, cultured guinea pig macrophages did not produce RNI in response to mycobacteria (Table 3.19.) and were able to kill M. tuberculosis B1453 in the presence of the iNOS inhibitor, N^G(MMA (Table 3.22.). Using the guinea pig as the animal model for human TB therefore leads to the conclusion that RNI are not likely to be involved in human macrophage tuberculocidal activity. As the majority of researchers fail to demonstrate the generation of RNI by human macrophages in vitro (Table 3.23.; section 1.9.2.), it would appear that, in the case of RNI, the guinea pig provides a more accurate picture of human macrophage antimycobacterial activity.

Acid-extracted lysosomal fractions of alveolar macrophages from BCG-vaccinated guinea pigs possessed tuberculocidal activity (Table 3.9.). It therefore urgently needs to be established whether M. tuberculosis is exposed to the lysosomal contents of activated guinea pig macrophages or whether the bacterium avoids exposure, as in non-activated murine and human macrophages (Armstrong and D’Arcy Hart, 1971 and 1975; McDonough et al., 1993; Clemens and Horwitz, 1995). Killing of tubercle bacilli by the lysosomal extracts took place at pH5.5 but not pH7 (Table 3.9.). It has been observed that mycobacteria inhibit the acidification of phagosomes in non-activated macrophages (Crowie et al., 1991; Sturgill-Koszycki et al., 1994; Xu et al., 1994). Thus, the pH of phagosomes in activated macrophages infected with M. tuberculosis also needs to be determined. Once these experiments have been performed, the importance of the lysosomal contents in macrophage tuberculocidal activity will be realised.

Even though there was no indication in vitro, it is still possible that guinea pig macrophages produce RNI for defence against mycobacteria in vivo. The generation of RNI in vivo can be detected by measuring the concentration of nitrate in urine (Stuehr and Marletta, 1987) and oral administration of N^G(MMA can be used to block this nitrate excretion (Granger et al.,
The levels of nitrate in the urine of guinea pigs infected with *M. tuberculosis* and the effect of N\(^{G}\)MMA on the survival of guinea pigs infected with *M. tuberculosis* should be ascertained. These experiments would determine whether RNI are produced when guinea pigs are infected with *M. tuberculosis* and whether the generation of RNI is an antimycobacterial mechanism. It could not be definitely concluded that any increase in nitrate excretion that occurred on mycobacterial infection was due to the production of RNI by macrophages. However, as macrophages are believed to be the principal effector cells in *M. tuberculosis* infection (section 1.7.), it seems likely that the induction of iNOS would take place inside macrophages and that they would be responsible for the generation of RNI in vivo.

In addition, it needs to be ascertained if ODC activity increases transiently when guinea pig macrophages are infected with *M. tuberculosis*. If an increase does occur, then it would be valuable to determine if a short-term elevation in ODC levels leads to the generation of large amounts of aminoaaldehydes for tuberculocidal activity. Unfortunately, a technique for measuring the concentration of aminoaaldehydes within macrophages does not yet exist. The principal problem is the highly reactive nature of aminoaaldehydes.

Although evidence gained from *in vitro* models is valuable, there is always the reservation that the behaviour of the macrophages in the *in vitro* model may not be comparable to the behaviour of macrophages *in vivo*. It is in this area that the technique of passaging mycobacteria through animals (section 1.10.) may prove useful. The susceptibility of the recovered inoculum to toxic agents reflects the mechanism(s) used by the host to kill the infecting mycobacteria, allowing conclusions to be made that are more relevant to the *in vivo* situation.

In this study, passaging *M. tuberculosis* H37Rv through the mouse selected for a population of mycobacteria that was more resistant to hydrogen peroxide *in vitro* (Tables 3.28 and 3.29.) but not more resistant to nitric oxide *in vitro* (Tables 3.30. and 3.31.). This indicates that the murine immune response to *M. tuberculosis* involves the generation of peroxide but not nitric oxide. However, evidence gained from other studies would support the opposite view (section 3.5.). It has been shown that reactive nitrogen intermediates are generated by murine macrophages for tuberculocidal activity both *in vitro* (Denis, 1991a; Flesch and Kaufmann, 1991; Barrera et al, 1994) and *in vivo* (Chan et al, 1995) but that hydrogen peroxide is not
essential for in vitro antituberculosis activity (Flesch and Kaufmann, 1988; Chan et al, 1992). Thus, the results gained from toxicity tests on the passaged mycobacteria contradict previously established theories.

However, it cannot be assumed that the hydrogen peroxide that the bacteria were exposed to during passaging was being produced by murine macrophages as an antituberculosis mechanism. Neutrophils are known to be associated with the tuberculous granuloma (Papadimitrou and Spector, 1972; Silva et al, 1989), generate large amounts of peroxide during phagocytosis (Iyer et al, 1961) and are reported to be capable of killing M. tuberculosis in vitro (Jones et al, 1970; Brown et al, 1987). The release of hydrogen peroxide by these cells might therefore have selected for a population of mycobacteria more resistant to peroxide than the parent strain.

It is possible that the passaged mycobacteria had become more resistant to nitric oxide in vivo but were not expressing this increased resistance in vitro. This is the major disadvantage with animal passaging as the technique cannot detect virulence determinants that are not expressed in vitro. Thus, passaging failed to provide definitive evidence for the production of hydrogen peroxide and nitric oxide as murine macrophage tuberculocidal mechanisms. However, these results with the passaged strain of M. tuberculosis (section 3.5.) illustrate that in vitro models may not always be the most accurate picture of the in vivo situation. In future, it would be useful to passage mycobacteria through guinea pigs rather than the mouse. The results obtained from these experiments would also provide evidence for the mechanisms used by human macrophages to kill M. tuberculosis in vivo.

In addition to studying the tuberculocidal properties of activated macrophages, the ability of M. smegmatis to survive acid stress was investigated (section 3.6.). Mycobacterium smegmatis mc²155 was used as a model organism for M. tuberculosis in these experiments. It was found that incubating M. smegmatis in mildly acidic medium (pH5.0) allowed the bacteria to survive subsequent exposure to extreme acidity (pH3.5) better than bacteria that had not undergone adaptation at pH5.0 (Table 3.36.). Thus, it was possible to adapt M. smegmatis to lethal pH values by pre-exposing the cultures to slightly acidic conditions. This phenomenon was denoted as an acidification tolerance response (ATR), after Foster and Hall (1990). An incubation period of
between fifteen minutes and one hour was required at pH 5.0 for the mycobacteria to adapt (Table 3.37.) and protein synthesis was necessary to ensure the development of an ATR (Table 3.38.). However, further experiments are required to ascertain if the ATR of *M. smegmatis* consists of different components, as in *Salmonella typhimurium* (Fig. 1.3.).

Having demonstrated that *M. smegmatis* possesses an ATR, *M. tuberculosis* should now be tested for an adaptive response to acid. If an ATR effect was observed with tubercle bacilli, its role in virulence could be determined (section 3.6.). It can be hypothesised that an ATR would be an important factor in mycobacterial virulence because it would allow survival in low-pH environments, such as the tuberculous lesion (Dubos, 1953). Mycobacteria have been shown to inhibit the acidification of phagosomes in non-activated macrophages (Crowle *et al.*, 1991; Sturgill-Koszycki *et al.*, 1994; Xu *et al.*, 1994). It would be desirable to know the pH of the mycobacterial-containing phagosome in activated macrophages in order to ascertain if an ATR could protect mycobacteria during infection. It is possible that the expression of the ATR within the macrophage phagosome would protect the bacilli not only from the acidity but also from other toxic mechanisms of the macrophage, such as the generation of hydrogen peroxide (Leyer and Johnson, 1993).

The full import of the work done in this thesis with lysosomal extracts and the ATR could be appreciated if it was determined that, in activated guinea pig macrophages infected with *M. tuberculosis*, phagosomes were acidic and that phagosome-lysosome fusion took place. The lysosomal contents of activated macrophages would then be established as a tuberculocidal mechanism and the ATR would become a known virulence factor of mycobacteria. Thus, the nature of phagosomes and phagolysosomes in activated guinea pig macrophages infected with tubercle bacilli is an area that requires urgent research.
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