Effector mechanisms of human anti-mycobacterial immunity

by

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SYNOPSIS

A number of effector mechanisms thought to be involved in immunity to mycobacterial infections in humans were examined in this thesis. The details of the studies performed in each chapter can be summarised as follows:

Chapter 2

Different methods of determining *Bacillus Calmette Guerin* (BCG) and *Mycobacterium avium intracellulare* (MAI) viability based on colony forming unit (CFU) counting and radio-isotope labelling were comparatively assessed. These included radio-isotope labelling with [^{3}H]uracil, [^{3}H]uridine, [^{3}H]glycerol, and CFU counting, by both agar plate dilution, and microcolony counting in broth. The sensitivity ranges of the different techniques were determined in both macrophage-free and macrophage-treated systems and used to assess the anti-mycobacterial potential of human monocyte-derived macrophages following BCG/MAI infection. A combination of radiolabelling with [^{3}H]uridine, confirmed by CFU assessment using microcolony counting, was found to be the most sensitive and reproducible means of determining mycobacterial growth and survival respectively. These techniques were then utilised in subsequent studies to assess various effector mechanisms thought to be involved in immunity to mycobacterial infections in human and murine cells.

Chapter 3

The role of reactive oxygen intermediates (ROI) and cytokine activation in the killing or inhibition of growth of mycobacteria within human phagocytes, were separately examined in this study. To look initially at ROI effects, peripheral blood-derived neutrophils and monocytes were isolated from normal healthy control individuals and from Chronic Granulomatous Disease (CGD) patients. Such patients are known to have a defect in their cellular membrane NADPH oxidase pathway, resulting in a failure of their phagocytes to generate oxygen radicals. Neutrophils and monocyte-derived macrophages from these two groups were then infected with BCG/MAI, and bacterial growth rates in each cell type from both groups were determined. No difference in intracellular BCG/MAI growth was demonstrable within neutrophils or macrophages, derived from either CGD patients or normal healthy controls. The results suggest that ROI are not important mediators in the intracellular control of mycobacterial infections within human phagocytes. Further studies were then undertaken to examine the effect of various cytokine treatments, known to activate mycobactericidal activity in murine cells, to affect BCG growth/survival within infected human monocyte-derived macrophages. The results revealed that macrophage activation with either, recombinant human GM-CSF, IFN-γ, TNF-α, or IFN-γ and TNF-α together, for between 4 to 7 days post-infection with BCG, had no demonstrable effect on bacterial growth or survival, as compared to non-stimulated control cells. The results then suggest that other

activation signals may be required to stimulate mycobactericidal activity in human macrophages.

Chapter 4

The role of tumour necrosis factor alpha (TNF-α) in macrophage-mediated killing of mycobacteria was also investigated in human cells. Four agents, thalidomide, oxpentifylline, dexamethasone and a polyclonal anti-TNF-α antibody, were all shown by specific ELISA to block endogenous TNF-α production by BCG-infected human monocyte-derived macrophages *in vitro* culture. There was however no significant enhancement of intracellular BCG growth, over a 7-day incubation period, in infected human cells, in the presence of any of the four TNF-α blocking agents, as determined by both radiometric and CFU counting methods of assessing bacterial growth and viability. The results suggest that the action of TNF-α alone is unlikely to be an important effector mechanism in antimycobacterial immunity within human cells.

Chapter 5

Five strains of *M. smegmatis* with different catalase expression/activity, were used to investigate the role of nitric oxide (NO) as an anti-mycobacterial mediator within murine and human macrophages. The strains: mc^2155 (wild type parental), BH1 (spontaneous, INH-resistant mutant of *M. smegmatis* mc^2155 which is deficient for catalase-peroxidase production), BH1/pBH4 (original shuttle plasmid), BH1/T35 ($katG^+$), & BH1/T586 (partially overlaps katG but does not carry the catalase-peroxidase gene from *M. tuberculosis*), were used to infect both the murine macrophage cell line J-774 and human peripheral blood mononuclear cells (PBMC)-derived macrophages. The levels of nitrite (NO_2^-) in the cell culture supernatants, and bacterial survival/growth, following infection were then determined. None of the five strains of *M. smegmatis* were observed to be killed within activated murine or human macrophages, as assessed by colony forming unit (CFU) determination and radiometric labelling, although high levels of nitrite (40-70nMol/ml) were detected in murine but not human cell culture supernatants. Nitrite titres in J774 supernatants moreover were not found to differ significantly following infection with the different catalase expressing strains. Possible catalase-mediated resistance to NO killing was also evaluated by assessing strain survival within cell-free cultures, containing differing concentrations of sodium nitrite ($NaNO_2^-$) or sodium nitroprusside (SNP), as *in vitro* generators of NO. The growth of the different strains of *M. smegmatis* were not affected by up to 10mM of either sodium nitrite or nitroprusside. The results suggest that mycobacterial catalase activity does not appear to affect either bacterial survival within murine or human macrophages, nor NO synthesis by infected cells. Additional results are also detailed, showing that BCG are similarly unaffected by physiological levels of $NaNO_2^-$ *in vitro* cell-

free culture, and that L-NMMA, an analogue of L-arginine, which has been shown to block NO-synthase (NOS), and hence NO production in murine cell systems, had no effect on the growth of BCG in human macrophage cultures. Overall therefore, the lack of detectable mycobacterial inhibition or killing in the presence of high levels of NO in murine systems, either synthesised intracellularly or generated *in vitro,* or in human cells following blocking of NOS, suggest that the NO pathway is unlikely be involved in controlling mycobacterial infections within either murine or human macrophages.

Chapter 6

Human $CD4^+$, mycobacteria-specific, cytolytic T-cell clones were used to lyse BCG-infected macrophages, and the effect on the subsequent growth and viability of the organisms was examined. The survival of released bacteria following cell lysis was assessed by both [^{3}H] uridine labelling and colony forming unit (CFU) estimation. The results indicate that even when effective antigen-specific or lectin-mediated cytolysis of the infected macrophages was achieved, there was no evidence for a direct mycobactericidal effect on the intracellular bacteria. This remained the case even if the period of co-culture of T cells and macrophages was extended up to 48 hours. Pre-treatment of the macrophages with IFN-γ was not able to act together with T cell-mediated lysis to produce inhibition of mycobacterial growth. Furthermore, no demonstrable effect on bacterial growth or viability was observed following re-phagocytosis of released BCG, by fresh human monocytes.

Chapter 7

A number of TB patients were identified in 7 family clusters with more than one individual with clinical TB. These families were considered as good candidate subjects to look for expression of cellular dysfunctions which might correlate with genetic markers of susceptibility. Monocyte-derived macrophage cultures were prepared from infected and non-infected family members and normal healthy controls. Mycobacterial growth rates, following a standard infection with BCG were then assessed for each subject's macrophages, and the levels of TNFα produced, on stimulation of uninfected cells with IFN-γ and/or LPS were also determined. The results show that in one Caucasian family of 14 in which 8 members in three generations developed tuberculosis, macrophages from 7 out of 8 diseased subjects, permitted high rates of intracellular growth, while 5 out of 6 asymtomatic family members exhibited low rates, comparable to those in unrelated healthy controls subjects. In a further 45 individuals from 6 Asian multicase families however, no such correlation was demonstrable between individuals with putative susceptibility to TB, and higher bacterial growth rates. Furthermore, no correlations were observed in any of the multicase families between the disease status of the subjects and TNFα production by their macrophages following IFN-γ and/or LPS stimulation *in vitro.*

Dedication

Praise be on Allah [The Lord of heavens and earths], and Muhammad, His messenger [peace and mercy be upon him].

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T. B, or not TB, that was the the question. I do not know much about the factors modulating intracellular BCG survival, but my survival would not have been possible without opsonization to quite a few people namely, Drs. Aqeel Ahmad, Augustine Akinwolere, Manjit Rahelu, M. Zurita, M. Kamal and Mark Hazelwood for their first hand advice and for cheering me up during my work. Pam Drysdale, B. Bartlett, and Christine Burt for tolerating me in the lab with a smile. Professor Alasdair Geddes, Professor Ian MacLennan and David Catty for my inter-departmental survival. David Purdham, Freda O'Reilly, Lin Brazier, Chandra Raykundalia for their help. Not to forget all the rest of my friends in the Departments of Immunology and Infection. Incidentally if I have left out somebody, that's purely intentional!

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LIST OF ABBREVIATIONS

Ab	Antibody
ADC	Albumin, Dextrose, Catalase
Ag	Antigen
BCG	Bacillus Calmette Guerin
CFU	Colony-forming unit
CM	Complete medium
Con A	Concanavalin A
cpm	Counts per minute
CTL	Cytolytic T lymphocyte
DTH	Delayed-type hypersensitivity
E/T	Effector-to-target cell (ratio)
ELISA	Enzyme-liked immunosorbent assay
FCS	Foetal calf serum
HBSS	Hank's balanced salt solution
HIV	Human immunodefeciency virus
HSP	Heat shock protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilodalton (mol. mass)
LPS	Lipopolysaccharide
Mφ	Macrophage
MHC	Major histocompatibility complex
ND	Not determined
NK	Natural killer (cell)
OADC	Oleic acid, Albumin, Dextrose, Catalase
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PPD	Purified protein derivative of tuberculin
TB	Tuberculosis
U	Unit

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CHAPTER 1

General Introduction

I.1 THE ORGANISM

1.1.1 Historical perspective

Tuberculosis (TB) has been recognised as a specific disease entity since antiquity, having been first described in ancient Indian writings of 2000 BC. Lesions caused by it have been observed in Egyptian, Inca and early stone age mummified remains [Zimmerman, 1979]. The Greeks and Romans also recognised TB, a clinical description of which was given by Hippocrates in 400 BC [Cave, 1939].

The earliest description of Miliary Tuberculosis was made in 1700 AD by John Jacob Manget and in 1819 AD the French Pathologist Rene Laennec was the first to describe the morbid anatomy and physical signs of the disease. Laennec was also the first to suggest the concept of tuberculosis being one disease involving many organ systems. In 1865 AD the French Physician Jean Antoine Villemin showed that TB was an infectious disease by producing lesions in rabbits and cattle infected with tuberculous material. However it was not until 1882 that the German microbiologist Robert Koch first isolated and identified the causative agent of TB, and succeeded in growing the bacillus in culture. In 1922 the BCG (*Bacillus Calmette Guerin*) vaccine was first used to immunise children against TB. The French microbiologists Albert Calmette & Camille Guerin produced the stable attenuated *Mycobacterium bovis* isolate following cultivation for over 13 years involving over 230 serial transfers on bile–glycerol-potato medium.

The first anti-tuberculous drug streptomycin was discovered in 1944 but its use was limited due to the rapid development of bacterial drug resistance to it, if used alone. In 1949 however the effective dual therapy of using both streptomycin in combination with paraminosalicylic acid (PAS) was published. This was later refined when the anti-tuberculous properties of Isoniazid were reported in 1952. This led to the established procedure of prescribing the three drugs in combination, to prevent the emergence of drug resistant strains.

The most outstanding recent advance in the effective treatment of TB however has been the discovery of Rifampicin in 1965. Rifampicin in combination with Isoniazid is still currently the most effective treatment of TB available. The striking increase of mycobacterial infections accompanying the AIDS epidemic and

more recently, the emergence of multi drug resistance strains of mycobacteria, has led to a new upsurge in research into tuberculosis. It is hoped that a better understanding of the mechanisms of immunity to mycobacterial infections may help to identify and develop new, effective treatment strategies for the future.

The recent application of monoclonal antibody and recombinant DNA technologies in mycobacterial studies has shed new light on the immunology, diagnosis and pathogenesis of mycobacterial infections. Cloned mycobacterial genes have been selected by monoclonal antibodies and expressed in *E. coli,* and the gene products have been purified and used in dissecting the host immune response to these well defined antigens to assess their potential role in protection and pathogenesis.

Chronological history of mycobacteria.

5000 BC	Mesopotamia and Egypt (Mummified human remains had recognised TB)
2000 BC	Indian writings describing tuberculosis.
400 BC	Hippocrates described pulmonary and spinal TB.
/	
/	
1700	John Jacob Manget described Miliary TB.
/	
1820~	Laennec disclosed the physical signs and morbid anatomy of TB.
1868	Villemin showed that the infection was caused by a transmissible agent.
1873	Gerard Armauer-Hansen identified *M. leprae*.
1880	Robert Koch discovered *M. tuberculosis.*
1882	Robert Koch described infectious nature of Tuberculosis.
1884	Cottage sanatorium for TB patients started in U.K.
1895	Roentgen discovered diagnostic radiography which then allowed the recognition of cavity formation in tuberculosis patients.
1922	*Bacillus Calmette–Guerin* (BCG) was first used as an antituberculous vaccine in humans.
1944	Waksman discovered the first anti-TB drug - streptomycin.
1949	Combination of streptomycin and PAS delayed emergence of streptomycin-resistant strain of *M. tuberculosis.*
1952	Isoniazid discovered, and two drug oral regime started.
1960	Most TB sanatoria in U.K closed.
1961	Ethambutol replaced PAS.
1966	Rifampicin launched.
1972	Short course TB therapy started.
1982	AIDS epidemic and resurgence of TB.
1991	First reports of multi-drug resistant strains of TB occurring in humans.

1.1.2 The list of approved species of Mycobacteria (1980)

M. africanum

M. asiaticum

M. aurum

*M. avium**

M. bovis

*M. chelonei**

M. chitae

M. farcinogens

*M. flavescens**

*M. fortiutum**

M. gadium

M. gastri

M. gilvum

*M. gordonae**

M. haemophilum

M. intracellulare

*M. kansasii**

M. komossense

*M. leprae**

*M. xenopi**

M. lepraemurium

M. malmoense

*M. marinum**

M. microti

*M. nonchromogenicum**

*M. neiaurum**

M. parafortuitum

*M. phlei**

*M. scrofulaceum**

M. senegalense

M. simiae

*M. smegmatis**

M. szulgai

M. terrae

M. thermoresistibile

M. triviale

*M. tuberculosis**

*M. ulcerans**

*M. vaccae**

* Names marked thus are very well established and are unlikely to change [After Skerman, McGowan & Sneath,1980, in Ratledge C., Stanford, ed. 1982]

1.1.3 Morphology

Mycobacterium tuberculosis is one of the most important species of the Mycobacteriaceae family. The bacilli are typically very slender (0.4-0.5μm x 3-5μm), curved often banded or beaded rods. They may occur singly, in pairs joined together at an angle or in clumps. Virulent strains form characteristic serpentine cords when grown in culture. They are obligate parasites that do not form spores. They can be classified as weakly gram positive .

Electron micrographs show that beyond the cytoplasmic membrane of the cell there is a rigid layer of murein Figure 1. Three discernible layers are superimposed upon it. These layers are derived from interconnecting structures stretching outward from the basement muramyl peptidoglycan as predominantly arabinogalactan mycolate with some arabinomannan and some peptidolipid, the rope-like structures of peptidoglycolipid terminate in ribbons at the cell surface. Mycolates of trehalose, including sulpholipids and cord factors are interspersed in these same outer areas [Barksdale and Kim, 1977].

Layers of mycobacterial cell wall (after Barksdale & Kim, 1977)

1. Mycosides (surface glycolipid/peptidoglycolipid)
2. Surface lipids
3. Mycolic acid (fibrous rope-like structure)
4. Cell membrane

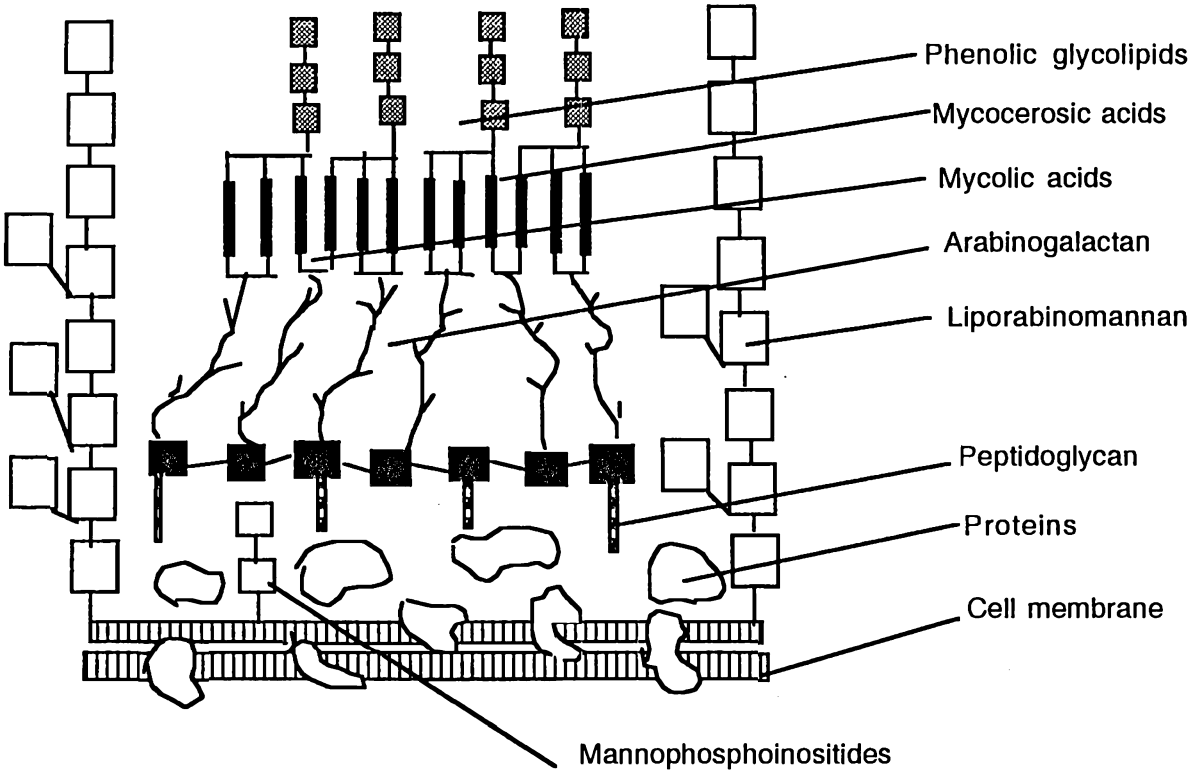


Figure 1.1

Cell wall of mycobacterium

I.2 THE DISEASE

1.2.1 Epidemiology

Humans are the main reservoir of the tubercle bacilli in nature. Currently the world population is approximately 5 billion and the numbers of persons infected with *M. tuberculosis* have been estimated to be at least 1 billion, [Snider, 1989]. The majority of the infected individuals carry their infection throughout life without developing the disease, of those who are exposed 75% remain un-infected and 20% of those infected, actually develop the disease [Snider, 1989]. Up to 50% of people become tuberculin positive in highly endemic areas of the world, 8 million new cases are diagnosed and 3 million die each year in developing countries [Styblo, 1989]. By the year 2000, an estimated 10 million people will freshly acquire active disease annually, of which one third will die [Ehlers *et al,* 1994]. Epidemiological, clinical and histopathological evidence suggests that whites are less susceptible than blacks [Crowle & Elkins, 1990, Stead *et al*, 1990] however evidence for differences among various human races in tuberculosis resistance lacks adequate controls [Dolberg *et al,* 1991]. In developing countries the risk of infection has declined as a whole but increased in certain minority groups [Grange, 1984]. In industrialised nations, excess cases have occured since 1984 mainly in the 25-44 yrs old members of marginalised groups. The incidence of tuberculosis in several African countries and its resurgence in the West has been attributed to the HIV/AIDS epidemic [Kushigemachi *et al,* 1984]. A coincident HIV disease is thought to account for approximately 14% of the surge by the end of century. The continuing increase in mycobacterial disease is due mainly to poplulation growth in areas with poor hygiene and low socioeconomic resources. New born infants irrespective of race remains especially susceptible to tuberculosis. Trends of immigration also influence incidences of infection. It is highest in Asian immigrants and lowest in North Western Europe and the USA [Enarson *et al,* 1979].

**Main risk factors for tuberculosis*

1.	Living in or originating from a developing country in which TB is endemic.
2.	Age (under 5 years; middle aged and elderly men)
3.	Alcoholism and/or drug addiction
4.	HIV infection
5.	Diabetes
6.	Lodging house dwellers
7.	Immunosuppression(corticosteroids, cytotoxic drugs)
8.	Close contact with smear-positive patients
9.	Silicosis
10.	Poverty and/or malnutrition

*After Ormerod, 1991

1.2.2 Clinical features

Infection generally occurs via the aerogenic route, and the lung is the prime site of infection and disease [Ratcliffe & Pallidino, 1953]. Nevertheless, any other tissue site can be infected after reactivation of dormant foci and hematogenic/lymphogenic dissemination.

**The time-table of tuberculosis*

Time post-infection	*Clinical features*
3-8 weeks	Conversion to tuberculin sensitivity
3 months	Miliary disease, tuberculous meningitis
3-6 months	Pleural effusions, segmental pulmonary lesions
1 year	First appearance of post-primary pulmonary lesions, Renal lesions, bone and joint disease.

*After Wallgren, 1948

Primary pulmonary tuberculosis is usually symptomless, and discovered only on routine screening of contacts. A chronic cough is the principal symptom, but other symptoms include fever, reduced appetite, and, in children, failure to thrive. Primary pulmonary tuberculosis is a mild illness which may resolve spontaneously without sequelae. Infants and in children under 5 may have serious immediate consequences.

Post-primary tuberculosis varies in its course. People from the Indian subcontinent, and Africa particularly, show an acute caseating lesion with gross cavitation. At the other extreme the disease may be chronic, grumbling on for many years. The clinical and laboratory presentation may be insidious and non-specific. They include malaise, weight loss, fever, night sweats, anxiety, depression, anorexia, and dyspepsia. Cough is the commonest symptom and in progressive disease sputum tends to be purulent and blood stained. Physical signs are often absent. The diagnosis of tuberculosis depends upon available expertise and is based on a combination of four criteria : clinical observations, radiological signs, microscopic examination of sputum and culturing the organism. The culturing and typing of mycobacteria may take upto two months. It is estimated that a sputum sample must contain a minimum of 10^4 mycobacteria for a trained microbiologist to detect acid fast bacilli on a direct sputum smear. Once diagnosed most patients are now treated on an outpatients basis, with or without a short initial period of hospitalisation. The duration of treatment, at one time lifelong, has now been reduced to only 6-9 months. Treatment of tuberculosis however requires compliance with therapy for at least 6 months.

1.2.3 New methods of diagnosis

1.2.3.1 Radiometric Method

In this method, the clinical specimen is inoculated in Bactec (Johnston Laboratories Inc, Townson, MD) radiometric broth. The medium itself is Middlebrook 7H12 medium with ^{14}C-radiolabelled palmitic acid as substrate [Rossman, 1992]. As mycobacteria metabolise the fatty acid, they produce $^{14}CO_2$, which is collected in the Bactec instrument for measurement of radioactivity, which is then recorded as a growth index [Good & Mastro, 1989]. With this method, the time required for isolation and identification of *M. tuberculosis* is decreased from 6 to 3 weeks, with an additional 5 to 7 days needed for drug susceptibility

testing [Good & Mastro, 1989]. an isolation-lysis centrifugation system is used for mycobacterial blood cultures to increase isolation of mycobacteria in the Bactec broth [Yamaguchi & Rechman, 1991].

1.2.3.2 DNA probes

Nuclear probes may be aimed at mycobacterial DNA or RNA. The sensitivity is increased several thousand times by using a probe to detect RNA rather than DNA, as the quantity of ribosomal RNA is much higher than DNA [Good & Mastro, 1989]. The DNA probe selected for commercial development is aimed at ribosomal RNA (Gen Probe, San Diego, CA). Three ^{125}I-labelled, single stranded DNA probes are available and are relatively specific for *M. tuberculosis* complex, *M. avium* and *M. intracellulare* [Rossman, 1992]. The probe to *M. tuberculosis* complex was 100% sensitive and 99.1% specific in tests of cultures of mycobacteria [Good & Mastro, 1989]. An estimated 10^5 to 10^6 cells are required for detection by Gen Probe; therefore this method cannot be used directly on clinical samples, and identification may be delayed by at least 3 weeks until cultures are available. Identification itself, however, takes only 4 hours [Good & Mastro, 1989]. Using a DNA probe and the Bactec medium, Ellner *et al*, (1988), claimed to identify 21% of the mycobacteria within 1 week and 66% within 2 weeks. Nuclear probes are expensive and have short half lives due to decay of radioactivity.

1.2.3.3 ***DNA Amplification by Polymerase Chain Reaction***

DNA amplification by polymerase chain reaction (PCR) is an exciting new development in the diagnosis of mycobacterial diseases. Three cloned sequences of *M. tuberculosis* have been described by Eisenach *et al*, (1990), that are promising as clinical probes. According to Eisenach *et al*, (1990), PCR product can be detected from purified DNA, equivalent to one cell, if the reaction was extended to 30 cycles. PCR has also been used to test clinical specimens with success [Brisson-Noel *et al*, 1989, Patel *et al*, 1990]. If this success is confirmed by further clinical studies, PCR may prove to be extremely useful in the rapid diagnosis of tuberculosis.

1.2.3.4 Serologic Tests

This is an area of intense research [Daniel, 1989, Good, 1989]. Monoclonal antibodies against *M. tuberculosis* have been developed. These can be used in ELISA assays for identification of mycobacteria grown in culture. The ELISA test is relatively inexpensive; however, at this time, the sensitivity and specificity are modest. With development of more specific monoclonal antibodies against *M. tuberculosis*, this may become a valuable method in the diagnosis of TB in the future [Rossman, 1992].

1.2.3.5 *Detection of Tuberculostatic Acid*

Tuberculostatic acid (TbA) is a long chain, branched, saturated fatty acid found in mycobacteria. It is also found in actinomycetes but not in other bacteria or human tissues. TbA can be identified by gas chromatography or mass spectroscopy, even in smear negative specimens, e.g., cerebrospinal fluid. For the identification of species, however, culture is still required. TbA also is expensive [Rossman, 1992].

1.2.4 Multi-drug-resistant (MDR) tuberculosis

Increasing outbreaks of multi-drug-resistant (MDR) tuberculosis have been reported during the last few years [Bloom & Murray, 1992, Iseman & Madson, 1989]. Approaches used to combat MDR infections included hospitalisation of new cases for several months as well as the introduction and successful application of Rifampicin (RIF) containing short course chemotherapy (SCC) to both new patients and surviving chronic cases. Certain developing countries achieved satisfactory results with SCC and drastically reduced MDR-TB by adequate treatment of cases [Kim, 1992, Chaulet *et al*, 1990]. There are two types of resistance of tubercle bacilli to antituberculous drugs; 'acquired' and 'primary resistance'. Resistance to at least one anti-TB drug is defined as 'acquired resistance'. This arises during, or after the course of treatment, usually as a result of faulty prescription or lack of adherence to the recommended regimen. Currently this type of resistance represents poorly functioning TB control [Spinaci, 1992]. A TB patient who has never received prior treatment if he develops drug resistance to at least one anti-TB drug, it is termed 'primary resistance'. It is caused by infection with drug-resistant organism, many of whom acquired

resistance due to inadequate treatment [Weyer, 1991]. The spread of MDR-TB strains has become most obvious in AIDS patients who, because of their increased susceptibility to infection and short interval between infection and manifestation of disease, represent early indicator cases. It is thought that HIV epidemic may produce increased levels of both acquired and primary resistance. When HIV-infected persons, even those with AIDS, are treated for TB disease, their ability to recover is no worse than that of non-immunocompromised patients as long as the therapy course is extended [Hopewell, 1992].

1.2.5 HIV and tuberculosis

The likelihood that an HIV-infected person who is infected with *M. tuberculosis* will develop clinical disease due to TB is 100 times greater than that of the general population with normal host resistance [CDC, 1992]. In HIV-infected persons, tuberculosis occurs in 30% of carriers of *M. tuberculosis* [Reider *et al*, 1989]. It has also been found that diagnosis of acquired immunodeficiency syndrome (AIDS) usually follows or coincides with tuberculosis [Perronne *et al*, 1992]. Since 1987, extrapulmonary tuberculosis has been included in the criteria for AIDS. The presence of pulmonary tuberculosis is one of the recommended markers for classification of clinical stage 3 in AIDS, whereas extrapulmonary tuberculosis is the recommended diagnostic criterion for clinical stage 4 disease. Diagnosis of tuberculosis is often delayed in HIV-infected patients. Clinical features of their illness may be atypical of TB and the episode of TB may precede other episodes of opportunistic infections [Hopewell, 1992], and it may mimic, and be mistaken for, *Pneumocystis carinii* pneumonia [Barnes *et al*, 1992b]. HIV-infected patients with tuberculosis who develop clinical disease are likely to do so within a relatively short time i.e. within weeks to months after they are infected with TB, as compared to persons without immune compromise who develop years after [Hopewell, 1992]. Compromised immune status due to HIV infection may reduce the effectiveness of anti-TB therapeutic regimens, thus increasing the chance of developing acquired drug resistance [Nunn *et al,* 1992]. Dually infected patients who get MDR-TB have a very high progression to frank disease (30% per year) as well as a high mortality rate (80%). In these cases progression from disease to death occurs very rapidly in as little as 4 months [Bloom & Murray, 1992, Iseman & Madsen, 1989].

1.2.6 BCG vaccine

BCG is an attenuated form of *M. bovis,* which was obtained on repeated culturing and constitutes a live vaccine against tuberculosis. *M. bovis* may cause disease in humans occasionally in a pulmonary form in children but more often causes infection of other tissues. Strains vary in their biochemical and virulent properties [Goldstein, 1982]. Between 1906-1919 the BCG vaccine was derived and first tested on humans in 1921. It is still the most widely used vaccine in the world with >3 x 10^9 doses having been given in the last 3 decades. The efficacy of BCG vaccine and its impact are controversial [Fine, 1989]. The present policy of WHO is to vaccinate newborns with BCG as an integral part of the Expanded Programme of Immunisation [Pio, 1989]. Currently BCG coverage is provided to 71% of the world population. In Europe 76% of the population is immunised with BCG. However Canada and the USA still doubt its efficacy and are reluctant to use BCG because it interferes with tuberculin testing as a method for detecting recent TB infection [Fine, 1989]. Live BCG vaccine may be unsuitable for HIV-positive individuals and is contraindicated in patients with clinical AIDS. The efficacy of BCG vaccine in the prevention of tuberculosis varies from 0-80% [Chaparas, 1980]. The failure of BCG vaccination in South India has provoked much attention [WHO bulletin, 1980]. Tools for gene transfer between mycobacteria have now been developed that should facilitate studies on mycobacterial pathogenesis and help in the development of better recombinant vaccines [Snapper *et al*, 1990].

1.2.7 Pathogenesis

Although other routes of transmission i.e. tonsils, intestinal tract and skin, have been documented, the most common is the aerial route [Grange, 1984]. Droplet nuclei of 1-5, μm in size are expelled during coughing, speaking and sneezing, and are inhaled. Mucociliary secretions eliminate the larger particles, and gastric acid destroys the ingested ones, when swallowed [Well, 1934, 1955, Ratcliffe & Pallidino, 1953]. A patient with highly active disease may cough over 10^9 bacilli daily. Only a minority of patients however are infectious to others [Riley, 1961], small droplet nuclei of 1-3 bacilli reach alveolar spaces, [Riley, 1961, Well, 1934].

An inflammatory reaction is initially induced with an influx of neutrophils [Bloch, 1948, Montgomery & Lemon, 1933] followed by monocytes. At the same time, the chemotactic factors released from the inflamed sites attract more monocytes from the blood. In the early lesion, there is little cell death or tissue necrosis and the tubercle bacilli multiply in the macrophages in a state of symbiosis [Dannenberg, 1989]. The main lesion is localised in the pulmonary cavity with approximately 10^8 colony forming units (CFU) of mycobacteria. In lung alveoli, free mononuclear phagocytes and T-cells exist in the external milieu and migrate back into tissues [Reynolds, 1991]. The immune mechanisms involved in human tuberculosis are more complex and, exclusively focused on the granuloma where a labile balance between T-cell mediated immunity and mycobacterial persistence determines the type of granuloma formed and the fate the infection will take [Dannenberg, 1991]. The primary lesion begins with an exudative form which then develops into a productive granuloma under the influence of lymphocytes. This granuloma has a relatively high cellular turnover. Freshly immigrant monocytes mature into epitheloid cells and multinucleated giant cells which harbour mycobacteria. CD4+ T lymphocytes predominate which induce tuberculostasis activities in phagocytes via IFN-γ, probably via collaboration with TNF and vitamin D3 [Denis, 1991a, Rook *et al*, 1987]. Productive granulomas are surrounded by a peripheral mantle of CD8+ T cells [Cooper *et al*, 1989]. Lysis of incapacitated phagocytes by CD4+ and CD8+ T cells may harm intracellular bacilli [DeLibero *et al*, 1988], or release bacteria to be taken up by more competent phagocytes. A role for γ/δ T cells in the early development of granuloma, in phagocyte attraction and accumulation may exist [Inoue *et al*, 1991, Modlin & Bloom, 1989].

Caseum is avascular, and adjacent blood vessels are thrombosed. It has a moderately acidic pH, a relatively low O_2 tension, and usually a relatively high concentration of bacillary components. Thus, macrophages cannot eliminate the tubercle bacilli within the caseous focus. Fortunately, for the same reasons, bacillary growth is also inhibited. Smaller caseous foci then undergo fibrous encapsulation but larger foci may reactivate if the host immunity becomes impaired. The hydrolytic enzymes of the macrophages seem to cause liquefaction of the caseous centre which then serves as an excellent culture medium for extracellular growth of *M. tuberculosis*. Tissue necrosis causes erosion of the nearby bronchi and they rupture forming a cavity. The bacilli are thus exposed to the air and spread to different parts of the lung.

In the majority (90-95%) of instances, this defence is successful in rendering the bacilli inactive or dormant [Stead, 1989]. Macrophages may however ingest the bacilli and transport them to regional lymph nodes. Also organisms may escape beyond the regional lymph nodes and reach the circulation and be disseminated hematogenously. Bones, meninges and organs of the genitourinary tract appear to be particularly frequent sites for the establishment of distant foci of infection [Daniel, 1980]. Most lesions heal but remain a potential foci of later reactivation. Cell mediated immunity (CMI) develops 2-8 weeks after primary infection. Healing then occurs, often with late calcification of the granulomas. The combination of a calcified peripheral lung lesion and calcified hilar lymph node is called the "Ghon complex". At this stage, some bacilli enter the blood stream and may then lie dormant in distant sites (notably the upper zones of the lung, the active epiphyses of long bones, the renal parenchyma), but are capable of producing clinical disease many years later. The infection follows this self-limited course in over 90% of normal cases, because the cellular immune response is sufficient to control the multiplication of the bacilli. In some individuals, progressive primary TB (e.g. miliary disease) occurs because the immune response fails to control multiplication of the bacilli. This occurs in less than 10% of normal adults, but is common in children under 5 years of age and in immunocompromised patients.

1.2.8 Genetic factors in tuberculosis

Based on population studies and animal models, genetic factors have been considered to play an important role in the susceptibility to mycobacterial diseases as in many other diseases, [Lurie *et al*, 1952, Rich, 1951, Dubos, 1964, Orme, 1987, Heinzel *et al*, 1989]. Susceptibility to tuberculosis in humans is multifactorial involving major histocompatibility complex (MHC) and non MHC-linked immune response genes. The HLA system, which is the MHC in man, is a major genetic constituent associated with susceptibility to diseases. The HLA system consists of two different sets of polymorphic genes, the Class I and Class II genes. The Class I genes contain three different loci, A, B, C and the Class II genes are composed of loci, DP, DQ and DR, except for DP, these genes are so close that they are usually inherited together or as a so-called haplotype. The Class I gene products are present on cell membrane of all nucleated cells, while the Class II gene products are mainly located on the cell surface of B cells, macrophages and activated T cells. A major function of MHC Class I and Class II molecules is the presentation of antigen to T cells. CD4+ T

cells recognise antigenic peptides in the context of MHC class II molecules and CD8+ T cells recognise antigenic peptides in the context of class I MHC molecules.

1.2.8.1 Genetic susceptibility in mice:

Lurie developed inbred rabbit families that were genetically resistant or susceptible to tuberculosis [Lurie *et al*, 1952]. Studies in mice provided further evidence of genetic factors linked to susceptibility to tuberculosis [Gros *et al*, 1981, Skamene, 1989]. A single autosomal dominant gene, 'Bcg' , on the centromere of chromosome 1 of mice confers resistance to infection by intracellular pathogens [Schurr *et al*, 1989, 1990]. The Bcg gene exists in two allelic forms; Bcg^r (resistant) and Bcg^S (susceptible). The resistance is linked to a T-cell-independent enhanced state of macrophage priming for bactericidal activity. The innate resistance operates at the level of macrophage, genetically susceptible mice develop T cell-mediated responses to control bacteria. The macrophages from resistant strains also have increased MHC class II expression and induce better stimulation of T cells. This effect was found to be eliminated by destroying macrophage function, i.e. chronic injection of silica. Recently, a candidate bcg resistance gene, denoted as 'N' ramp (natural resistance associated macrophage protein) has been cloned [Vidal *et al*, 1993]. The human homologue to the Bcg gene is being actively sought [Blackwell, 1989].

1.2.8.2 Genetic susceptibility in humans:

Genetic factors may also be very important in humans in determining whether an infection develops into disease and also on the course of the disease [Mims, 1987]. The contribution of environmental factors like geographical distribution, poverty, exposure, low socio-economic conditions however makes the data difficult to interpret. The Lubecks disaster provided evidence to suggest that innate resistance and susceptibility to tuberculosis did occur in humans [Mims, 1987]. In this incident a group of babies was inadvertently immunised with a virulent strain of tubercle bacilli in a small town in Germany, causing a third of them to die. The remainder suffered only minor lesions which later resolved. Race moreover also appears to be a variable in susceptibility to tuberculosis. In macrophages from blacks intracellular replication is higher than from white counterparts and blacks are twice as likely to be infected by

Mycobacterium tuberculosis than whites [Stead, 1990]. This resistance at a cellular level appears to be a composite of innate resistance, presumably due to ingestion and destruction by unstimulated macrophages and T cell-dependent acquired immunity.

In experimental systems it has been shown that genes which determine immune responses are closely linked with MHC genes. MHC-linked immune response genes influence T-cell responsiveness and susceptibility to tuberculosis. Immunogeneity of different mycobacterial epitopes vary according to the MHC phenotype of the host. Immunisation of inbred strains of mice with soluble extracts of TB bacilli in adjuvants revealed H-2 linked control of antibody levels to mycobacterial proteins [Ivanyi *et al,* 1988]. Moreover immunoblotting showed distinct antibody response patterns in the various strains examined [Lindquist, 1986]. Some association has also been reported between HLA class I allelles and tuberculosis, but they vary within the population [Selby, 1978, Al-arif *et al,* 1978, Hafez *et al,* 1985]. In China and India co-seggregation of pulmonary tuberculosis with HLA-DR2 haplotype has been reported from family studies [Singh *et al,* 1983]. A significant association between sputum +ve tuberculosis and DR2 and immune responses to 38kda protein of *M. tuberculosis* have also been described [Bothamley *et al,* 1989]. Sixty percent of the mouse genome is conserved in man on long arm of human chromosome 2. The pattern of resistance seen to BCG is also observed in other intracellular infections such as, *Leishmania donovani* and *Salmonella typhimurium* [Vidal *et al,* 1993].

1.3 THE IMMUNE MECHANISMS

The immunology of mycobacterial infections is complicated and has not been completely understood [Edwards & Kilpatrick, 1986, Ellner & Wallis, 1989]. A defective cell mediated response in the initial contact with *M. tuberculosis* has been postulated as a cardinal predisposing factor in the progression of active tuberculosis. The tubercle bacilli become coated with C3b generated via the alternative pathway and mononuclear phagocytes then take up bacteria by phagocytosis following binding to CR3 and CR1 complement receptors [Schlesinger & Horwitz, 1990, Jones, 1974]. The bacteria can then lodge and multiply in vesicles (phagolysosomes) within the cytoplasm [Crowle, 1988]. Uptake via Fc receptors (FcR) however induces ROI generation and hence may contribute to antimicrobial resistance. In contrast, uptake

via complement receptors (CR) causes endocytosis in the absence of ROI production and thus facilitates bacterial survival. *M. tuberculosis/M. bovis* supports its own phagocytosis by mononuclear phagocytes via two mechanisms;

1. Secretion of abundant quantities of fibronectin binding molecules of 30-32 kDa and 57-60 kDa which promote phagocytosis by fibronectin receptors [Abou Zeid *et al*, 1988, Borremans *et al*, 1989].
2. Induction of C3 activation followed by deposition of C3 products and uptake via CR [Schlesinger & Horwitz, 1990]. This entry then by-passes reactive oxygen intermediates induction.

Virulent *M. tuberculosis* strains have been proposed to evade the killing mechanisms of macrophages by escaping from fused phagolysosomes either into nonfused vesicles or into the cytoplasm [McDonough *et al*, 1993]. Macrophages then play a crucial role in generating an immune response, by phagocytosing and processing antigen for presentation to T helper lymphocytes. The macrophages and other antigen presenting cells then mediate antigen induced activation of T cells of the helper-inducer phenotype. Sensitised T-lymphocytes and various mycobacterial components activate macrophages which become more motile and increase the number of lysosomes and release of lysosomal enzymes and toxic O_2 radicals. Sensitised T-lymphocytes on encountering processed antigens from TB bacilli accumulate at the infection site, and release lymphokines. Macrophages are then transformed into epithelioid cells which may fuse together to form giant cells. They are surrounded by a cuff of lymphocytes mainly CD4+ T cells which induce fibroblasts to produce collagen under the influence of cytokines. The role of epithelioid cells is mainly secretory and they contain numerous vesicles. Within a few days, and before onset of the acquired immune response, a typical concentric tubercle is formed. The initial response provokes an immune response that is usually capable of limiting the proliferation of the organisms. This immune response also appears to provide the host with a high degree of immunity to re-infection. However in most infected individuals the organisms remain dormant, but capable of reactivating and causing disease at a later time. Thus clinically apparent disease can result from either the early progression of the initial infection (so-called primary disease) or from later reactivation of a latent form of infection (post-primary disease).

1.3.1 Humoral immunity

The immune response to the presence of *M. tuberculosis* is indicated by the appearance of agglutinins, precipitins, opsonins, and complement-fixing antibodies in the serum. The first mycobacterial contact or infection is said to determine the pattern of immune-response to all subsequent reactions [Abrahams *et al,* 1970]. The primary humoral immune response to *M. tuberculosis* consists of short-lived synthesis of IgM followed by a predominance of IgG antibodies [Diene *et al,* 1968, Daniel *et al,* 1967]. IgA antibody is known to elevate in extensive disease but the most pronounced specific antibody which correlates with active disease is said to be IgG [Benjamin & Daniel, 1982, Kalish *et al,* 1983, Gibson, 1987]. The mycobacteria, being a complex of antigenic components, evokes a specific humoral response to a wide range of antigens. Humoral immunity has been shown to be protective against many infecting organisms, but unfortunately, the humoral response to *M. tuberculosis* fails to play an important role. There is indirect evidence that patients with hypogammaglobinemia i.e. low antibody levels do not show a high incidence of tuberculosis than individuals with normal antibody levels. In some cases, serum from immune animals may even enhance the growth of mycobacteria [Forget *et al,* 1976]. Moreover the efforts to make use of the humoral response to devise sero-diagnostic tests logistically comparable and reproducible as microbiological methods, have not been successful due to antigenic cross reactivity of *M. tuberculosis* antigen with those of other mycobacteria and other bacterial species.

1.3.2 Cell mediated immunity (CMI)

Delayed-type hypersensitivity (DTH) reactions such as the tuberculin test are expressions of the cell-mediated immunity [Lefford, 1975] which plays a key role in halting proliferation of *Mycobacterium tuberculosis in vivo* [Mackaness, 1969]. Koch observed that re-injection of guinea-pigs with tubercle bacilli, after they had recovered from a previous infection, resulted in the development of an indurated non-necrotic lesion reaching maximum size in 24-48 hours at the site of the second inoculation. These lesions subsided thereafter and the bacilli did not spread systemically as they did in the first inoculation, but localised in enlarged lymph nodes. This phenomenon, which is also commonly observed following second exposure to other infectious agents both bacterial and viral, is recognised as delayed hypersensitivity (DTH).

It differs from immediate hypersensitivity by the time interval between re-exposure to antigen and appearance of the symptoms, and in being mediated by lymphoid cells [De Weck, 1977]. In humans, the prototype DTH is the tuberculin reaction which is characterised by erythema, induration, and histologically by infiltrates of neutrophils and mononuclear cells. The mononuclear cells consist of monocytes and small to medium-sized lymphocytes, with the majority of cells having the characteristics of activated macrophages, only a small proportion of the cells are T lymphocytes [Chase, 1945]. The antigen recognition of DTH requires the stimulation or activation of a specific subpopulation of T lymphocytes by antigen presenting cells probably macrophages. This activation of T cells causes a release of cytokines.

It is generally accepted that resistance to Mycobacterial infections depends on the complex interplay of two main cell types, phagocytic cells and T cells. A specific response occurs when T lymphocytes recognise bacterial antigens on the surface of antigen presenting cells of the phagocyte family. These include monocytes, macrophages and dendritic cells of lymph nodes. The invading organism is trapped and internalised by phagocytes situates near the primary site of entry, usually the lung. If the organism is phenotypically hardy and capable of initiating a primary infection it begins to grow in this intracellular environment. During this growth phase and/or due to damage inflicted by phagocyte anti-bacterial functions, microbial products become accessible to processing and presentation on the surface of the infected cell. In contrast to antibodies which bind antigen directly, T cells see partially degraded antigenic peptides in combination with self major histocompatibility determinants (MHC) [Livingston & Fathman, 1987]. After phagocytosis by macrophages, bacterial products are processed and presented in combination with MHC class II determinants, which are then able to elicit an immune response. T lymphocytes which recognise processed antigen are subsequently activated, proliferate and differentiate into effector cells. This process is commonly termed clonal selection.

Current dogma assumes that phagocyte anti-microbial effector mechanisms are enhanced by phagocyte activation. Bacterial, phagocyte and T cell derived chemotactic factors attract further T cells and macrophages to leave the blood stream and accumulate at the inflammed site [Kaufmann, 1993]. Although some macrophage activation occurs in the absence of sensitised, antigen reactive T cells, the most important mechanism is thought to involve lymphokine release by such T cells localised within the lesion.

Macrophages are themselves able to produce lymphokines which help to attract and activate freshly migrated monocytes (IL-1, GM-CSF, MCSF).

Macrophages harvested from BCG-vaccinated rabbits, infected *in vitro* with live *M. tuberculosis* and then replaced in the anterior chamber of the eye were better at inhibiting the growth of BCG than were macrophages harvested from control unvaccinated animals. These findings were confirmed by other workers and shown to be true for other organisms. The term "activated macrophage" was first used by Mackaness, initiated by his pioneering work on Listeriosis in mice [Mackaness, 1964, Mackaness, 1962]. Mackaness observed that the macrophages increased their ability to destroy listeria, this was coincident with substantial morphological changes within the macrophage and the development of a DTH reaction [Mackaness, 1968, Blanden *et al*, 1969]. These observations linked macrophages and T cells in protection against intracellular pathogens.

1.3.3 T lymphocytes in tuberculosis

The occurrence of a specific DTH reaction to mycobacterial antigens demonstrated that the host responded to these organisms in a specific manner. Specific antibodies had already been demonstrated, but adoptive transfer of protection or DTH could not be achieved by specific antibody. The cellular nature of DTH was formally shown by transferring contact sensitivity to picryl chloride in guinea pigs with live peritoneal exudate cells [Hahn & Kaufmann, 1981]. Tuberculin hypersensitivity was transferred in a similar manner [Hahn & Kaufmann, 1981]. Some degree of non-specific protection to listeria was demonstrable when cells from BCG vaccinated donors were injected in combination with BCG. Since there was no protection when cells alone were injected these experiments provided further evidence that antigen specific sensitisation was required for transfer of immunity [Mackaness, 1964].

Additional evidence for the role of T lymphocytes in experimental infections in mice was provided by the use of anti-lymphocyte serum, direct injection of which was able to suppress resistance to *M. tuberculosis*, *M lepraemurium* [Gaugas & Rees, 1968] and *L. monocytogenes* [Mackaness, 1964]. Experiments with

congenitally athymic [Ueda *et al*, 1976] thymectomised and irradiated mice confirmed these findings and clearly established a pivotal role for T cells in the control of intracellular infections.

1.3.3.1 T-cell subsets

T cell immunity plays an important role in intracellular infections where antibodies fail to act [Hahn & Kaufmann, 1981]. Infected host cells usually present microbial antigens on their surface in association with host structures encoded by the major histocompatibility (MHC) gene complex; this feature allows the identification of infected cells by T lymphocytes rather than by B lymphocytes or antibodies [Zinkernagel & Doherty, 1979, Fitch, 1986]. Upon antigen-specific interaction with infected host cells, T lymphocytes are activated to become the central mediators and co-ordinators of immunity to intracellular microbes. Both protection to and pathogenesis of intracellular bacteria are therefore linked with T lymphocytes. The T cell system has important protective functions which act independently of antibody-induced effector systems. T cells are derived from the thymus and can be divided into 2 major subpopulations on the basis of their cell membrane glycoproteins which can be defined with monoclonal antibodies.

T cells are critical in the development of cell-mediated immune reactions and are essential for acquired immunity to intracellular bacteria. Peripheral T cells exhibit extensive phenotypic heterogeneity. When T cells are activated, they are able to perform several different functions, such as helper function, suppressor function, and cytotoxicity, etc. On the basis of functional criteria, the T-cells system can be divided into three subclasses;

1. Helper T-cells; produce a variety of potent biological mediators-termed interleukins-and thus are capable of activating targets cells to perform their effector functions.
2. Cytolytic T-cells through direct cell-cell contact lyse their target cells.
3. Suppressor T-cells influence the biological functions of their target cells in a negative way done regulating cell activation, proliferation and cytokine production. The functional identity of these cells is less clear.

T cells are heterogeneous in nature and it has been shown that a T cell clone can express multiple functions. In contrast to antibodies which interact directly with free antigen, T lymphocytes recognise a processed antigen on the surface of host cells in the context of self molecules encoded by the MHC. All T lymphocytes express a heterodimer, which is responsible for antigen recognition and commonly termed the T-cell receptor, as well as the CD3 molecule, which is intimately associated with the T-cell receptor and involved in the transfer of activation signals following antigen stimulation [Fitch, 1986].

In mice, long-term T-helper clones can be divided into two types, i.e. Th1 and Th2 subsets, with distinct cytokine secretion phenotypes [Romagnani, 1991]. Th 1 cell types produce IFN-γ, IL-2, and TNF-β, Th 2 cell type produce IL-4, IL-5, IL-6, IL-9, IL-10 and CSF. Th1 produce more TNF-α and GM-CSF than Th2, [Mossman & Coffman, 1987, 1989].

1.3.3.2 CD4+ T cells

The relative contribution of CD4+ and CD8+ T cell subsets in protection against intracellular pathogens in experimental infections, has been studied by two complementary experimental protocols [Kaufmann, 1993]. Lymphocytes from immune donors can be negatively selected *in vitro* prior to transfer to a naive host which is then infected with the original organism, or animals can be depleted *in vivo* of T cells by using specific monoclonal antibodies prior to infection. *In vitro* depletion experiments were able to demonstrate that adoptive transfer of protection against *L. monocytogenes* was abrogated by complement mediated lysis of CD4+ and CD8+ cells prior to transfer [Kaufmann *et al,* 1979, Kaufmann *et al,* 1985], the CD4+ cells were responsible for the accumulation of phagocytes in the infected spleen [Czuprynski & Brown, 1987]. A similar protective effect of CD4+ cells has been shown in experimental murine *M. tuberculosis* infection, protection was abated by depletion of both CD4+ and CD8+ cells, greater effects were seen when CD4+ T cells were depleted [Orme & Collins, 1983].

The contribution of each subset appeared to vary dependent on the disease state; CD4+ cells were required early in infection when bacteria were rapidly multiplying within macrophages, while CD8+ cells became important later in the infection but were then able to allow faster recovery in recipient animals [Orme,

1987]. Other groups have provided clear evidence that CD4+ cells are required for protection. Muller *et al* (1987) found that depletion of CD4 cells by *in vivo* treatment with specific antibody in mice, led to higher levels of infection with *M. tuberculosis* in the spleen, compared with similar treatment with CD8 antibodies. Depletion of CD8+ cells also led to increased bacterial cell numbers [Muller *et al*, 1987]. In another study of BCG infection in mice, CD8+ cells were found to have an insignificant effect on the course of infection, whereas removal of CD4+ cells led to a dramatic increase in the number of organisms isolated from the spleen and liver of infected animals [Pedrazzini *et al*, 1987]. Contrary examples showing a clear involvement of CD8+ cells in experimental systems have also been reported.

These animal studies have highlighted the importance and synergistic relationship of T lymphocyte subsets in infections with intracellular bacteria. It seems that CD4+ cells are of major importance but a role for CD8+ cells cannot be excluded [Muller *et al*, 1987]. *In vitro* studies where experimental conditions can be more closely controlled and monitored have been able to clarify some of the more elaborate mechanisms of anti-bacterial immunity. Of particular relevance in this respect is the ability to produce defined T cell lines and clones. T cell clones have been generated from mice immunised with killed *M. tuberculosis* which were able to recognise mycobacterial antigens in a MHC restricted fashion [Kaufmann & Flesch, 1986]. All clones were able to produce IL-2 and IFN–γ after antigenic stimulation. Supernatants from these antigen activated clones were able to inhibit the proliferation of BCG within macrophages *in vitro* and resulted in reduced numbers of BCG harvested from macrophages [Flesch & Kaufmann, 1987]. In addition clones were able to transfer DTH against PPD in normal mice as measured by footpad swelling. Other mycobacteria reactive clones producing IL-2, IFN–γ and GM-CSF (granulocyte-macrophage stimulating factor) have also been demonstrated [Mustafa *et al*, 1986].

1.3.3.3 Cytolytic CD4+ T cells

Helper activities of CD4+ cells have been well characterised, in contrast to their cytolytic ability, possibly due to its relatively recent discovery. PPD was shown to induce CD4+ CTL from peripheral blood mononuclear cells from mantoux positive donors [Hank & Sodal, 1982]. These cells were able to help alloreactive cytotoxic cells mature and express a cytolytic phenotype against the stimulating cells. In

addition to providing T cell help, these PPD responding cells were also cytolytic in their own right and were able to lyse autologous target cells in an antigen dependent manner. Since then, there have been many reports of CD4+, class II restricted, CTL with the ability to lyse macrophage [Kumararatne *et al*, 1990, Hansen & Kristensen, 1986] and B cell targets [Kumararatne *et al*, 1990]. Class II restricted CTL specific for other mycobacterial [Kumararatne *et al*, 1990, Kale *et al*, 1990] proteins have also been reported in human and murine systems [Tite & Janeway, 1984, Vyakarnam *et al*, 1988, Sinohara *et al*, 1988]. This cytolysis is dependent on direct contact between the T cell and the target cell and is not dependent on soluble mediators. Induction of apoptosis in the target cell appears to be a major mechanism of such cytotoxicity [Rahelu *et al*, 1993]. It is quite likely that T-cell mediated cytolysis contributes to immunopathology in tuberculosis and leprosy. The relevance of CD4 mediated cytolysis to protection is unclear. *In vivo* induction of cytotoxicity within skin lesions of lepromatous leprosy, by injection of IL-2, or PPD appears to result in a reduction of bacterial load [Kaplan *et al*, 1988].

Many of the studies undertaken with cytolytic CD4+ T cells have been carried out using cloned cells or long term T cell lines [Tite & Janeway, 1984, Spits *et al*, 1982, Vyakarnam *et al*, 1988], as well as short term cultured blast cells [Kumararatne *et al*, 1990, Hansen & Kristensen, 1986]. CD4+ CTL have been difficult to demonstrate in freshly isolated samples and usually require *in vitro* stimulation to express a cytolytic phenotype. However, injection of PPD into skin lesions of lepromatous leprosy patients led to destruction of infected macrophages, by immigrant CD4 and CD8 T cells. These bacteria are released into the extracellular environment [Kaplan *et al*, 1988], since *M. leprae* is an intracellular pathogen such a mechanism is potentially beneficial. In addition, MHC class II expression is inducible in *M. leprae* infected human Schwann cell cultures, which may therefore be able to present antigens to CD4+ cells [Samuel *et al*, 1987].

A negative immuno-regulatory role has also been postulated for CD4+ CTL in which lysis of antigen presenting cells results in removal of the stimulus and so terminates the immune response [Braakman *et al*, 1987, Lanzavecchia *et al*, 1988]. In one study BCG reactive CD4+ T cell clones were able to suppress their own BCG induced proliferation while enhancing the proliferation of other clones from the same donor [Sinohara *et al*, 1988]. This may be an example of antigen specific immune suppression.

Studies in murine models [Orme, 1993] have provided a clear insight into the kinetics of protective immunity and the generation of immunological memory. An initial wave of IFN-γ producing CD4+ cells, which preferentially recognise infected macrophages or those presenting secreted mycobacterial proteins, followed by Th2 type cytokine producing CD4+ cells which react to constitutive components of mycobacteria as well as culture filtrate proteins. The later emerge when the initial infection has been contained and most mycobacteria killed.

1.3.3.4 Cytolytic CD8+ T cells

A few studies in murine models of mycobacterial infections have suggested that CD8+ cells may play a role in immunity to tuberculosis. CD8+ T cells have been found to be weakly protective in adoptive transfer studies [Orme, 1987]. Also cytolytic CD8+ T cell clones, derived from mice immunised with killed *M. tuberculosis*. H37Ra, were shown to lyse autologous macrophages pulsed with either killed H37Ra or live BCG, a few of which were found to produce IFN–γ [De Libero *et al*, 1988].

Antigen-specific CD8+ T-cell clones with cytolytic ability have also been established from mice immunised with *M. leprae* [Chiplunkar *et al*, 1986]. *M. leprae* specific clones were able to lyse Schwann cell targets pulsed with *M. leprae* [Steinhoff & Kaufmann, 1988]. This may be of significance since Schwann cell destruction in leprosy is the major mechanism leading to the neurophilic states seen in patients.

Interest in a role for CD8+ T cells in tuberculosis was revived recently when it was reported that β-2 microglobulin mice succumb to progressive, disseminated disease after being given a sublethal dose of *M. tuberculosis,* but not BCG infection [Flynn *et al*, 1992]. Such mice are unable to generate CD8+ T-cells due to a targeted disruption of the β-2 microglobulin gene [Flynn *et al*, 1992], providing the strongest evidence yet for an important role for CD8+ T-cells in immunity to tuberculous infections. Indirect evidence may also be inferred for such a role, in that mice with a targeted disruption of the gene encoding IFN–γ also develop fatal disseminated disease when infected with sub-lethal infections of *M. tuberculosis* [Orme, 1993].

It is important to remember that such experimental evidence is derived exclusively from murine infections, and although crucial in developing theories of acquired resistance to intracellular pathogens, experimental infections may differ markedly from human ones. Mice have no natural *M. tuberculosis* infection, moreover mycobacteria-reactive CD8+ T cell clones and lines have proven relatively difficult to generate in man [Rees *et al*, 1988].

Evidence for a decisive role for CD8+ CTL in the control of human mycobacterial infections remains limited. Indeed, to date, only one report has been published which suggests a possible protective function in man, in which It was found that a patient with recurrent tuberculosis, who had both low CD8+ and CD4+ T-cell numbers before treatment, was found after initial, non-effective treatment, to have normalised CD4+ levels but low CD8+ cells and in whom, CD8+ numbers only recovered after prolonged effective treatment [Bothamley *et al*, 1992].

It is clear however that humans are able to control an infection with *M. tuberculosis* , since the majority of persons from prevalent areas do not succumb to a clinically relevant disease, yet display tuberculin positivity. How these bacteria are killed *in vivo*, is still uncertain. A strong humoral response to TB infections can occur, but on its own or in combination with complement seems unable to destroy the invading organisms.

Tubercle bacilli are remarkably competent against the formidable arsenal presented to them by macrophages. Once phagocytosed, they appear able to inhibit the formation of potentially destructive phagolysosomes [Gordon *et al*, 1980] and even if phagosome-lysosome fusion occurs they seem able to resist the chemical attack that ensues. Unlike other intracellular pathogens they are highly resistant to an oxidative burst, and activation of human macrophages by lymphokines *in vitro* has not yet produced bacteriostasis or killing sufficient to explain human immunity.

Protective and pathogenic situations involving CTL (both CD8+ and CD4+) can be imagined however. Lysis of infected macrophages may have a direct detrimental effect on intracellular tubercule bacilli. The mechanism whereby this occurs remains to be determined but evidence for such a scheme has been

presented in murine and human *in vitro* systems [Pithie *et al*, 1992, Kumararatne *et al*, 1990, Kale *et al*, 1990].

Bacterial cell death may result from release of bacteria from quiescent tissue macrophages into freshly immigrant, activated monocytes which are better able to kill bacilli, or bacteria may be killed by accumulated damage inflicted from repeated phagocytosis and lytic release [Kaufmann, 1989, Kaplan *et al*, 1989]. Conversely a pathogenic consequence of this lysis is also possible: if the bacteria are not killed as a result of macrophage lysis, then it may contribute to the tissue destruction which is seen in TB patients [Pithie *et al*, 1992]. In addition, release of bacteria from their restricted locale could lead to dissemination around the body and via the airways to new hosts. Cytolytic T cells have also been shown to release TNF which may also prove toxic to surrounding tissues and has been shown to be elevated in the pleural space of TB patients.

In man, mycobacterial antigen-specific cytolytic T-cells have been generated from the peripheral blood of BCG-vaccinated subjects [Pithie *et al*, 1992, Kumararatne *et al*, 1990] from patients with leprosy [Kale *et al*, 1990] and from those with tuberculosis [Kumararatne *et al*, 1990]. There has been much speculation, therefore, about the function of cytolytic T cells in the immune response to tuberculosis.

1.3.3.5 Natural killer (NK) -cells

Natural killers cells are a subset of lymphocytes that are able to lyse target cells without prior sensitisation or histocompatibility restriction [Herbsman, 1987]. They are active in parasitic and bacterial infection [Nencioni *et al*, 1983, Klimpel *et al*, 1986], and have been shown to have high activity in tuberculous pleural effusions where they are activated through IFN–γ and IL-2 [Okubo *et al*, 1987].

1.3.3.6 Gamma-delta (γ/δ)T-cells

In humans γ/δ delta T-cells are present in blood, lymphoid tissues, and a variety of non-lymphoid organs, but with the exception of spleen, they represent less than 10% of the total numbers of T lymphocytes present

at these sites [Groh *et al,* 1989]. They express a TCR composed of γ and δ subunits [Raulet, 1989].The γ/δ cells recognise antigen in an MHC unrestricted manner and activated clones have been shown to produce cytokines that caused macrophage aggregation and proliferation [Haregewoin *et al,* 1989]. It has been postulated that they may possibly be the first line of defence against invasion by *Mycobacterium tuberculosis* [Hass *et al,* 1990, Munk *et al,* 1990] and have been shown to express antigen specific cytotoxicity *in vitro* [Janeway *et al,* 1988].

Some of their roles are summarised below:

1. Mycobacterial antigens preferentially stimulates the proliferation of γ/δ cells ['O Brien, 1989, Kabelitz *et al,* 1990, Havlin, 1991].
2. An extraordinarily high number of thymic and splenic γ/δ cell hybridomas from mice were found to react with purified protein derivative (PPD) [O' Brien, 1989, O' Brien, *et al,* 1992].
3. There is an increase in activated CD3+ α β TCR negative T-cells in mice when they are exposed to aerosol extracts of *Mycobacterium tuberculosis* [Augustin *et al,* 1989].
4. There is a relative increase of γ/δ cells after primary exposure in the draining lymph nodes of mice immunised with *M. tuberculosis* but this effect is absent in subsequent exposures [Janis *et al,* 1989].
5. Local accumulation of γ/δ T-cells preceding α/β T-cells were noted after intra-peritoneal infection of mice with *M. bovis* on day 3 and 7 [Inoue *et al,* 1991].
6. The granulomatous skin lesions from patients of Leprosy show increase numbers of γ/δ T-cells following lepromin injection, and in reversal reactions, but not in tuberculoid or lepromatous lesions [Modlin & Bloom, 1989, Uyemura & Modlin, 1991].
7. Marked expansion of γ/δ T lymphocytes from the peripheral blood of normal healthy individuals by *in vitro* stimulation with *M. tuberculosis* preparations was observed [Delibero & Lanzavecchia, 1991, Kabelitz *et al,* 1990, 1991].
8. Increased number of γ/δ T-cells were also observed in necrotic areas of tuberculous lymphadenitis, with the majority of cells concentrated in the vaccinity of or inside necrotic regions [Falini *et al,* 1989].

γ/δ T cell stimulation has been proposed to be suggestive of the superantigen activity of bacterial toxins for α/β and γ/δ T cells [Rust *et al*, 1990, Herman *et al*, 1991]. A low mass (< 3 kDa), protease-resistant molecule stimulates the production of γ/δ T cells [Pfeffer *et al*, 1990]. The exact nature of this entity operationally functioning as superantigen however is not yet known, but indirect oligoclonal γ/δ T cell activation by mycobacteria has been put forward as an evidence. This is thought to result from interleukin induction of accessory cells or α/β T cells, which then cause selective stimulation of γ/δ T cells [Tsuyuguchi *et al*, 1991].

Biologic functions of mycobacteria-stimulated γ/δ T cells

1. Lysis of mycobacteria-pulsed target cells [Barnes, *et al*, 1992].
2. IFN-γ secretion [Follows, *et al*, 1992].
3. TNF-α secretion [Follows, *et al*, 1992]

Gene-knockout (gKo) mice model in immunity to mycobacterial infections

	Death due to infection with:	
gKo mice	***M. tuberculosis***	**BCG**
β_2 microglobulin	yes	no
CD4+	no	na
MHC classII	yes(temporary delayed)	yes
TCR-γδ	na	no(primary infection)
IFN-γ	yes	na
CD8+	yes	na

[Reviewed in Ehlers *et al*, 1994]

na = data not available.

1.3.4 Role of cytokines

T cell soluble factors or cytokines induce macrophage activation and have many other functions vital to mycobacterial immunity. In murine model systems cytokines have been shown to have many antimycobacterial effects but these are less convincing in human systems. A few important cytokines in relation to mycobacterial immunity are discussed.

1.3.4.1 Interleukin-1(IL-1)

All commonly used adjuvants are potent inducers of IL-1 in macrophages. Mycobacterial proteins and LAM induce IL-1 which is not dependent on the presence of the intact microbe. It mediates the non-specific or acute phase response, and plays an essential role in immune induction, facilitating T-lymphocyte expression of IL-2 receptors and release of IL-2. This process is critical to the expansion of antigen-specific T-lymphocytes and the subsequent elaboration of other cytokines with monocyte activating factor activity, such as IFN–γ which may be important for mycobacterial killing [Hamblin, 1988]. In *M. tuberculosis*/*M. bovis* infection IL-1 levels are increased [Wallis *et al*, 1990, Ogawa *et al*, 1991]

1.3.4.2 Interleukin-2 (IL-2)

It causes growth inhibition of AIDS associated strains of MAI in humans [Bermudez & Young, 1988] and has been found to result in the inhibition of BCG growth *in vivo* in mice. However no effect on mycobacterial growth has been shown *in vitro* in the mouse. There is indirect, morphological evidence in man that intralesional injection of IL-2 in lepromatous leprosy induces bacterial killing [Toba *et al*, 1989]. It has also been suggested that it is a useful marker of T-cell activity and disease progression in patients with TB [Christopher *et al*, 1991]. The reconstitution of cell-mediated immunity in cutaneous lesions of lepromatous leprosy by IL-2 has also been cited [Kaplan, 1989].

1.3.4.3 Interleukin-4 (IL-4)

It is capable of activating tuberculostatic functions in murine MP [Flesch & Kaufmann, 1990, Denis, 1991d]. Moreover in *in vitro* human cell systems it synergises with TNF-α or GM-CSF to inhibit *M. tuberculosis* and *M. avium* infections [Denis, 1990]. In mice it causes growth inhibition of *M. tuberculosis in vitro* in bone marrow derived macrophages, if infected before lymphokine treatment, but has no effect on peritoneal macrophages [Kaufmann, 1989]. IL-4 induces monocyte influx *in vivo* and formation of multinucleated giant cells *in vitro* [McInnes & Rennick, 1988] and in this way may contribute to formation and stabilisation of tuberculous granulomas.

1.3.4.4 Interleukin-6 (IL-6)

IL-6 is produced during *M. bovis* infection [Huygen *et al*, 1991], and its production by blood MP's derived from *M. tuberculosis*-infected subjects was found to be increased [Ogawa *et al*, 1991]. It causes growth inhibition of BCG *in vitro* in murine model systems if added before infection [Flesch & Kaufamnn, 1990]. It has been reported to increase the growth of a virulent strain of *M. avium* in human macrophages [Denis, 1992].

1.3.4.5 Interleukin-8 (IL-8)

IL-8 is produced by MP and other cells, and a recent study showed that IL-8 secretion by human MP lines following phagocytosis of *M. tuberculosis* [Freidland *et al*, 1992]. Its role in tuberculous infections needs further definition but it is thought to be involved in the development of TB lesions and in granuloma formation [Gronhoj *et al*, 1989, Gregory *et al*, 1992].

1.3.4.6 Granulocyte-monocyte colony stimulating factor (GM-CSF)

In vitro studies in human cells have shown that GM-CSF can cause growth inhibition of *Mycobacterium tuberculosis* at 100U/ml and growth inhibition of MAI at 10U/ml [Denis, 1990], but has no similar effect

on mouse cells. It is also thought to play a role in the attraction and activation of phagocytes early after infection [Gregory *et al*, 1992, Serushago *et al*, 1992].

1.3.4.7 Interferon-gamma (IFN-γ)

IFN–γ is produced by T lymphocytes from blood or lymphoid tissues upon stimulation with specific antigens, mitogens or alloantigens. All cellular mediators of resistance namely: $CD4^+$ and $CD8^+$ lymphocytes, gamma/delta T cells and natural killer (NK) cells can produce IFN–γ [Kaufmann, 1993]. It is an anti-viral and anti-proliferative protein which also has potent immunoregulatory effects including, activation of macrophages, resulting in induction of Class I and Class II major histocompatibility complex (MHC) antigen expression on both macrophages and other cells of non-haemopoietic origin, IFN–γ increases NK cell activity, causes activation and differentiation of other immune cells, and it induces the production of reactive oxygen species by macrophages [Nathan *et al*, 1983a, 1983b]. Recently it was shown that recombinant IFN–γ, when administered locally into lesions of leprosy patients [Nathan, *et al*, 1986], a strong infiltration of T cells and monocytes was elicited and in some cases led to decreased numbers of intralesional *M. leprae* organisms, and promotes granuloma formation at the site of application [Kaplan & Cohn, 1991].

Activation of murine macrophages by factors produced by T cells and r-IFN–γ has been reported to inhibit intracellular growth of mycobacteria. Flesch & Kaufmann [1988] used bone marrow-derived macrophages (BMM) cultured in serum-free medium for 9 days to achieve tuberculostasis where a variety of reactive oxygen metabolites failed to neutralise the effects of IFN–γ. Evidence of heterogenous susceptibility of *M. tuberculosis* strains towards IFN–γ -activated BMM was obtained when *M. tuberculosis* H37Rv was shown to be sensitive while *M. tuberculosis* Middleburg proved resistant [Kaufmann & Flesch, 1987, Flesch & Kaufmann, 1988]. IFN–γ also has variable effects on the intracellular growth of *M. tuberculosis* in human macrophages *in vitro* [Rook *et al*, 1986a, 1986c], however it enhances intracellular growth of virulent strains of *M. tuberculosis* [Douvas *et al*, 1985, Rook, 1990a] and *M. lepraemurium* [Mor *et al*, 1989]. In contrast, IFN–γ failed to directly activate mycobacterial growth inhibition in human monocytes [Crowle, 1988]. IFN–γ however has been identified in the pleural fluids of patients with tuberculous pleuritis

[Barnes, *et al*, 1990]. IFN–γ has also been hypothesised to exert its tuberculostatic effects indirectly by inducing synthesis of functionally active form of vitamin D3 from the circulation [Kaufmann & Flesch, 1987].

1.3.4.8 TNF-α

It is produced by many cell types including activated macrophages and T lymphocytes, and has a wide range of inflammatory, cytotoxic and cytostatic effects [Old, 1985]. It is a conical shaped trimer molecule composed of 157 amino acids and is 45-50A^0. TNF was purified from culture supernatant of stimulated human pro-myelocytic leukemic line, HL-60, [Wang *et al,* 1985] to give a non-glycosylated protein, with one disulphide link, and of molecular weight of 17kd and a pI of 5.3. The gene encoding for TNF is located on chromosome 6 in human within the MHC close to the HLA-B region [Kriegler *et al,* 1988]. Studies of mRNA levels and TNF-α production by cells support the concept that the control of TNF-α production occurs mainly at the post-transcriptional level [Kriegler *et al,* 1988]. Stimulants of TNF-α production; include *Mycobacterium tuberculosis*, BCG, Listeria, *E. coli* etc. [Valone *et al,* 1991].

Evidence is also accumulating that TNF-α is an important macrophage activating factor for antibacterial resistance against infections caused by facultative intracellular organisms. Prolonged release of TNF-α has been suggested to play a role in the cachexia observed in many chronic infections. The fever, weight loss, and tissue damage which are characteristic of TB may be attributable to the release of TNF. Macrophages in tuberculous granulomata are activated by IFN–γ and by an autocrine feedback loop involving calcitriol. The sera of tuberculous patients contains high levels of an inhibitor of TNF related to the extracellular domain of the TNF receptor, [Rook & Al-Attiyah, 1990d]. Membrane-associated TNF is involved in the activation of anti-leishmanial defence. TNF appears to be an important mediator of many inflammatory reactions. Activation of human polymorphonuclear neutrophils function by TNF-α and IFN-γ has also been cited [Shalaby, 1984].

Although TNF alone is incapable of activating tuberculostatic capacities in murine MP, it shows marked synergy with IFN–γ [Flesch & Kaufmann, 1988]. Furthermore, IFN–γ–induced tuberculostasis is blocked by

anti-TNF antibodies. Experiments *in vivo* in mice with neutralising antibodies have shown a protective role for TNF against BCG infection. It is found that the neutralisation of TNF by anti-TNF antibodies markedly interferes with the formation of granulomas and subsequent reduction of mycobacteria in BCG infected mice [Kindler *et al,* 1989]. It is proposed that TNF released from macrophages in the microenvironment of developing granulomas is involved in a process of autoamplification, acting in autocrine or paracrine way, enhancing its own production and release, thus favouring further macrophage accummulation and differentiation leading to bacterial death, [Rook & Al-Attiyah, 1990d]. Differences in TNF secretion by structurally different lipoarabiomannan have been associated with differences in virulence [Chatterjee *et al,* 1992].

1.3.5 Role of vitamin D3

In conditions where there are chronic TB granulomata in man, there are changes in vitamin D metabolism attributable to the activation within the macrophage of a I-hydroxylase which converts the inactive circulating form of vitamin D3 into calcitriol. Calcitriol is the most effective defined activator of antituberculosis activity in human monocytes [Rook, 1988] and in man it is probably an integral part of the macrophage activation pathway. Incubating the cells with one or the other of the two hormonal vitamins A and D makes them resistant to infection with virulent TB [Crowle *et al,* 1987, 1990].

Macrophages within granulomatous lesions convert $25(OH)D_3$ to the $1.25(OH)_2$ derivative. Activation of macrophages increases the synthesis of the active form of vitamin D3. It is the most potent activator of human and murine macrophages for the inhibition of the growth of *M. tuberculosis* [Rook, 1988]. Thus, In extreme cases, so much of the active metabolite is formed that it exerts not only local effects within the granulomata, but also spills out into the systemic circulation where it causes abnormalities of calcium metabolism [Rook, 1988].

A relationship between low vitamin D levels and increased susceptibility to tuberculosis has also been reported [Davies, 1985]. Moreover there is also evidence that vitamin D therapy cured skin TB infections in the 1940's [Macrae, 1947]. TNF-α and IFN-γ also have a synergistic effect on macrophages in the presence

of vitamin D3 [Denis, 1991a]. Exposure of macrophages to either IFN-γ or calcitriol *in vitro* enhances their potential for massive release of TNF [Rook, 1988]. Lower photosynthesis of vitamin D is believed to be a genetic factor that makes residents of Asiatic origin living in United Kingdom 20-30 times more susceptible to tuberculosis infections than white residents [Rook, 1985]. Dark skin reduces sunlight induced synthesis of vitamin D, thus inadequate dietary intake of vitamin D, reflected by low serum concentration leads to increase susceptibility to tuberculosis. Asians living in U.K have a diet that is deficient in vitamin D as compared to their counterpart West Indians living in the same climate resulting in low serum levels of vitamin D, which is associated with reduced cell-mediated immunity [Rook, 1988].

1.4 INTRACELLULAR SURVIVAL OF *MYCOBACTERIUM TUBERCULOSIS*

The term 'intracellular bacteria' commonly comprises bacterial pathogens endowed with the capacity to replicate inside cells of the host [Hahn & Kaufmann, 1981, Moulder, 1985, Kaufmann, 1993].

Markers of intracellular bacterial infections

1. Capacity to enter host cells, and the exploitation of intracellular survival mechanisms e.g. inside mononuclear phagocytes allow the bacteria to avoid antibody responses.
2. Promotion of T-cell response by processing and presentation of their proteins.
3. Representing tissue reaction as typical granulomas.
4. Chronicity of the disease due to successful containment inside granulomas.

Mycobacterium tuberculosis and *M. bovis* fulfil all the criteria. The use of intracellular habitat, as a niche effectively protects them from the humoral arm of the body's defence mechanisms. As a main pre-requisite for successful intracellular survival *in vivo,* mycobacteria have evolved certain properties to adapt to the adverse conditions inside host cells, especially mononuclear phagocytes (MP), which are preferred because they are highly phagocytic, long lived and possibly insensitive to TNF induced cell death. These strategies may include:

1. Invasion of non-professional phagocytes[*M. tuberculosis/M. bovis* rarely inhabit them].
2. The evasion into cytoplasmic compartment [McDonough, *et al*, 1993].
3. Inactivation of reactive oxygen intermediates by superoxide dismutase (SOD).
4. Inhibition of phagosome-lysosome fusion [Armstrong & Hart, 1971]
5. Neutralisation of phagosomal pH.
6. Mycobacteria produce certain substances like heat shock proteins, and virulence factors which co-ordinate gene regulation [Miller *et al*, 1989]. Elevated levels of hsp synthesis inside MP renders the cells more resistant to subsequent, otherwise lethal, hits.
7. Suppression of T cell function i.e. priming/activation by LAM (lipoarabinomannan). In the mouse, LAM induces TNF-α secretion by macrophages, and it has been recently shown that LAM from the virulent strain has the capacity to avoid the triggering of TNF-α and other early response genes responsible for anti-bacterial activities [Chatterjee *et al*, 1992, Roach *et al*, 1993].
8. The outer layer of sulfolipids inhibit phagosome-lysosome fusion and interfere with ROI production [Goren *et al*, 1976, Pabst *et al*, 1988, Zhang *et al*, 1988, Sibley *et al*, 1988].

The role of mononuclear phagocytes in intracellular mycobacterial infection is twofold because they represent both an essential habitat and the major effectors cells of the host defence system. They are however highly heterogenous with respect to their anti-mycobacterial capacity which depends on their tissue site and activation status stage. The major effector functions of monocyte/macrophage are following:

1. Generation of reactive oxygen intermediates (ROI)
2. Production of reactive nitrogen intermediates (RNI)
3. Increase heat shock proteins (hsp) synthesis.
4. Limitation of intracellular iron availability
5. Phagosome acidification and phagosone-lysosome fusion
6. Production of defensins.
7. Polyamines.

The first two mechanisms cited above are controversial, with evidence both for and against then being cited in the literature. Iron is critically required for intracellular bacterial survival. Limitations in the available intracellular iron by down regulation of transferrin receptors and/or decrease of intracellular ferritin levels may be an important macrophage defence medium. Defensins are basic polypeptides which are plentiful in granulocytes and some mononuclear phagocytes [Lehrer *et al,* 1991]. Purified defensins possess potent microbicidal activity, and polyamine oxidase-mediated killing of African trypanosomes has been demonstrated [Ferrante *et al,* 1982], but no such role have been reported in mycobacterial immunity [Kaufmann, 1993].

1.4.1 Anti-oxidant enzymes

All organisms from prokaryotes to eukaryotes contain superoxide dismutase (SOD) [Fridovich, 1978, 1986]. The function of which is to convert SOD dismutes superoxide radicals generated during respiration to hydrogen peroxide, which in turn is removed by catalase

The reaction is as follows:

$$2O_2^- + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \xrightarrow{catalase} O_2 + 2H_2O$$

Three types of SOD have so far been found;

1. Mn SOD [Normally present in bacteria]
2. FeSOD [Normally present in bacteria]
3. CuZn SOD Present in *Brucella abortus* [Beckman *et al,* 1990]

Some pathogenic mycobacteria secrete SOD [Kusunose *et al,* 1976], which may help intracellular bacterial survival. However *M. smegmatis*, an avirulent mycobacteria does not secrete SOD [Kusunose *et al,* 1976] . Recently it has been postulated that SOD, as well as other anti-oxidant enzymes, play an important role in protecting parasites from the host immune response. Parasites with high levels of antioxidants are then thought to be more resistant to oxidant damage. The bactericidal activity of γ-interferon-activated

macrophages is significantly blocked by SOD or catalase in the incubation medium. Hence measurement of SOD is thought to be not a good index of virulence of mycobacteria [Kaufman, 1993].

Both catalase and peroxidase are biochemically characterised and their activities known in some mycobacteria [Wayne & Kubica, 1986, Lygren *et al*, 1986]. The loss of catalase/peroxidase activity is thought to be responsible for isoniazid resistance [Zhang *et al*, 1992b].

1.4.2 Secreted proteins

Mycobacteria are known to secrete proteins during growth as demonstrated by the presence of proteins in culture filtrate of *in vivo* grown mycobacteria. The importance of mycobacterial secreted proteins in infection and development of protective immunity has also been reported [Rook *et al*, 1986b, Orme, 1987]. Moreover, since pathogenic mycobacteria live within macrophages, secreted proteins may be presented earlier to T cells than cytosolic proteins which are only available when they are broken down by macrophages. Antigen presentation of mycobacterial secreted proteins may be unique and it is not yet known whether antigen presentation occurs via the endogenous (Class I) or exogenous (Class II) pathway or both. Indeed, it has recently been shown that hemolysin, which is a secreted antigen of the intracellular pathogen *Listeria monocytogenes*, is presented via both the endogenous and exogenous pathways [Brunt *et al*, 1990]. The function of mycobacterial secreted proteins, on the part of mycobacteria seems to have evolved specifically for their nutrition and survival and thus may be involved indirectly in their pathogenesis.

1.4.3 Heat shock proteins (hsp)

Heat shock proteins of a pathogen can be induced upon entry into a mammalian host due to temperature rise and other stressful stimuli e.g. reactive oxygen species, produced by host phagocytes and also pI and pO changes, and Fe deficiency [Lathigra *et al*, 1990]. Nutrient deficiencies also induce heat shock responses, and this may explain why mycobacteria produce high amounts of 65 hsp when cultured in Zn^{++}-deficient medium [De-Bruyn *et al*, 1981]. Hsp's are highly conserved from bacteria to man with a homology of more

than 50%, and are major components of all living cells. They can be divided into several groups according to their molecular mass. Besides house-keeping functions hsp serve as major targets of the immune system [Young *et al*, 1987, 1988, 1990, Young & Mehlerts, 1989]. Among the handful of mycobacterial hsp's identified, one 65 hsp was invariably isolated by different laboratories [Young, 1990]. When monoclonal antibodies to mycobacterial antigens were screened against a variety of mycobacterial species, 14 different binding patterns were observed [Buchanan *et al*, 1987]. Some monoclonal antibodies reacted with all 23 mycobacterial species tested; one antibody was capable of distinguishing between *M. leprae* and the *M. tuberculosis/M. bovis* complex, and one antibody reacted exclusively with *M. leprae*. These findings indicate that the 65 hsp contain highly conserved regions shared by many if not all mycobacterial species, and at least one region which segregates the *M. tuberculosis/M. bovis* complex from *M. leprae* and from the most atypical mycobacteria, and lastly one epitope unique for the species *M. leprae*. Subsequent to these studies it was found that cross-reactive monoclonal antibodies to 65 hsp were not restricted to the genus *Mycobacterium* but also recognised a variety of other pathogenic and non-pathogenic, gram-positive and gram-negative bacteria [Young *et al*, 1987, Shinnick *et al*, 1987]. This even included *E. coli*, the species originally used for cloning of the gene. It thus became clear that the molecule contains species-specific, genus specific, and broadly cross-reactive epitopes [Lamb *et al*, 1989].

The 65 hsp was also found to correspond to the so-called common antigen, 62 kd, and the GroEL protein of *E. coli* [Young *et al*, 1988]. The GroEL protein is a member of the heat shock protein family which are synthesised in response to heat shock in order to protect the cell [Lindquist & Craig, 1986]. T-cell reactivity to the 65 hsp is frequently found in healthy individuals [Emmrich *et al*, 1986, Munk *et al*, 1988, 1989, 1990]. T cell clones with specificity for the 65 hsp are also isolated from tuberculosis patients [Oftung *et al*, 1987]. The primary structure of *M. leprae* and *M. tuberculosis* 65kD antigens are 95% identical [Mehra *et al*, 1986, Shinnick, 1987]. In addition, T cells have been described which recognise the region shared by mycobacteria and *E. coli* [Young *et al*, 1987].

10% of all the proteins of *M. bovis* are hsp 60 [De Bruyn *et al*, 1987]. Hsp 60 of mycobacteria shares about 60% sequence homology with its human counterpart [Jindal *et al*, 1989]. Sequence analysis of the *M. tuberculosis* 71kD molecule and the closely related 70kD antigen of *M. leprae* are identified as structural

homologues of Dnak protein of *E.coli* [Young *et al*, 1988], which is a member of a major family of 70kD heat shock proteins (hsp70) found throughout nature [Lindquist, 1986]. Cross reactivity is observed between mycobacterial 70/71kD hsp and its human homologue. The sequence of *M. leprae* 18kD antigen, a low molecular weight hsp, indicates a relationship with a further class of low molecular weight heat shock proteins from plants [Young *et al*, 1988].

1.4.4 Heat shock proteins and autoimmunity

65 hsp has been cited to be involved in the development of autoimmunity [Van Eden *et al*, 1988]. Immunisation of rats with complete Freund's adjuvant induces experimental arthritis [Van Eden *et al*, 1988]. In arthritis patients, augmented T-cell reactivity to *M. tuberculosis* has been described which may be caused by the 65 hsp, and arthritis has been suspected as a side effect of BCG therapy [Torisu *et al*, 1978, Holoshitz *et al*, 1986]. There is however no unequivocal demonstration of an association between arthritis and tuberculosis. The wide spread distribution of the 65 hsp correlates among micro-organisms, indicates that there is a common epitope shared by various pathogenic and non-pathogenic bacteria rather than that a single infectious agent may be involved in the induction of autoimmune responses. Lactofferrin, transferrin and DR (β) subsets of MHC class II molecules were shown to cross react with the 65kD protein of mycobacterial origin [Aguas *et al* 1990]. This cross reactivity may then contribute to trigger the autoimmunity. It has been found that synovial fluid T-lymphocytes of patients suffering from inflammatory arthritis showed more reactivity towards;

(i) Recombinant preparations of *M. bovis* 65kD hsp [Res *et al* 1988],

(ii) Purified protein derivative of tuberculin [Abrahamsen *et al*, 1978],

(iii) With an acetone fraction of *M. tuberculosis* [Holoshitz *et al* 1986; Pope *et al* 1991],

(iv) And with recombinant 65kD antigen of *M. leprae* [Gaston *et al* 1988; Gaston *et al* 1989].

The acetone precipitable fraction of *M. tuberculosis* is also rich in antigen cross reactive with cartilage proteoglycan [Holoshitz *et al* 1986].

1.4.5 The antigens of mycobacteria

Microbial entities which under appropriate conditions, induce a protective immune response against the viable pathogen are called protective antigens. These antigens when seen by protective T cells constitute candidate vaccine subunits. Many bacterial antigens are recognised by T cells from leprosy and tuberculosis patients, as well as by mice infected with *M. tuberculosis* [Orme *et al*, 1992, Schoel *et al*, 1992, Anderson *et al*, 1990]. When an organism is killed or degraded the structural antigens are thus presented to the immune system. On the other hand, secreted antigens are processed by alive and dividing intracellular bacteria, hence forming primary antigens, to be presented in the earliest stages of infection. It is reported that T-cell responses to secretory antigens is very strong, both in patients and *M. tuberculosis/M. bovis* infected mice [Orme *et al*, 1992]. Structural proteins are then presented as antigens primarily in chronic infections.

1.4.6 Molecular biology of the mycobacteria

Complex antigenic structure and slow rate of growth makes it difficult to obtain sufficient quantities of pure antigens or material from mycobacteria for immunological or biochemical studies. A combination of recombinant technology, monoclonal antibodies and synthetic peptide technique is having a great impact on mycobacterial research. Genomic libraries of mycobacteria have been constructed using different vector-host systems [Young *et al*, 1985, Clark Curtiss *et al*, 1985]. The most successful one is the λgt11 expression library system [Young and Davies, 1983]. Many genes of mycobacteria have so far been cloned, which have been selected for monoclonal antibodies and expressed in *E. coli*. The gene products moreover have been purified and used in dissecting the host immune response to well defined antigens, to assess their potential role in protection and pathogenesis in animal models [Colston, 1990]. Specific epitopes or antigens defined by monoclonal antibodies have also been identified that have been used to improve the serodiagnosis of mycobacterial disease [Jackett *et al*, 1988]. The recent development and application of the polymerase chain reaction (PCR) technique [Saiki *et al*, 1985] has shown great potential in the rapid diagnosis of mycobacterial diseases.

1.4.7 Mechanisms of mycobacterial growth inhibition

Both oxygen dependent and independent microbicidal effector mechamisms in anti-mycobacterial immunity have been cited [Elsbach & Wiess, 1983].

1.4.7.1 *Oxygen-dependent effector mechanisms*

Upon phagocytosis of micro-organisms, phagocytes become activated and O_2 consumption increases a 100-fold to generate O_2 species that exhibit microbicidal effects [Curnutte & Babior, 1987]. Monocytes and macrophages when activated by bacterial ingestion produce superoxide and hydrogen peroxide. Reactive Oxygen Intermediates (ROI) are highly reactive on a variety of cell constituents i.e. cell membrane, DNA, lipid, proteins and enzymes, [Basaga, 1990]. In the presence of NADPH and NADPH oxidase which converts molecular oxygen (O_2) to superoxide radical, which being unstable is converted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD). At low pH ferrous ion (Fe_2+) is released from lactoferrin, and more toxic radical (hydroxyl) (OH-) [Hasset & Cohen, 1989]. They cause functional and structural damage and even cell death.

O_2 ——————NADPH—————— O_2^-

NADPH oxidase |

Fe_2+ from lactoferrin

|

Cell components----------OH^- (more toxic than H_2O_2 and O_2-)

| [Hassett & Cohen, 1988]

|

Structural and functional damage [Basaga, 1990]

The myeloperoxidase pathway, which is present in neutrophils but not in macrophages, utilises H_2O_2 to form hypochlorous radical (OCl^-) in the presence of halides, such as chloride Cl^-. Since phagocytic cells

contain amines such as spermine and taurine, OCl^- can react with such amines to form chloramine derivatives that are long-lived and have microbicidal activity [Weiss *et al,* 1983]. However their role in killing pathways have not yet been described.

Indirect evidence for (Reactive Oxygen Intermediates) ROI:

i) Phagocytosis of micro-organisms causes increased O_2^- release from the phagocytes [Curnette & Babior, 1987].

ii) CGD patients, who have genetic defect in NADPH oxidase which is responsible for the production of ROI, suffer from recurrent bacterial and fungal infections [Curnette & Babior, 1987] and several cases of fatal disseminated BCG infection have been reported when CGD patients were given BCG vaccination [Kolayashi *et al*, 1984].

Direct evidence for ROI:

i) There is protection of *M. microti* by added catalase against killing by lymphokine-activated murine macrophages *in vitro* [Walker & Lowrie, 1981].

ii) A co-incidence is shown in infected guinea-pigs between immunologically-enhanced killing of hydrogen peroxide-susceptible mutants and enhanced release of H_2O_2 by granuloma macrophages [Jackett *et al*, 1980].

iii) A correlation is found between high microbial virulence in guinea-pigs and resistance to H_2O_2 [Jackett *et al*, 1978].

1.4.7.2 *Oxygen-independent effector mechanisms*

Oxygen independent killing mechanisms are also utilised by the phagocytes [Cartelli, 1983, Spitznagel & Shafer, 1985, Catteral *et al*, 1986, Murray, 1988]. This is composed of the following mechanisms:-

1. Phagosome-lysosome fusion.

   The granules and phagolysosomes of phagocytes contain cationic proteins [Rest *et al*, 1978], lysozyme, lactoferrin, and other digestive enzymes [Spitznagel & Shafer, 1985].

2. Nitric oxide pathway.

Indirect evidence for O_2-independent killing mechanisms:

i) Since ROI is poorly triggered upon entry of some intracellular pathogens into phagocytes, O_2-independent killing mechanisms have been considered to play an important role [Catteral *et al*, 1986, Rothermal *et al*, 1986, McCabe & Mullins, 1990].

   The following intracellular pathogens induce a poor respiratory burst following infection:-

   1. *M. leprae* [Hozer *et al*, 1986].
   2. *M. lepraemurium* [Mor *et al*, 1988].
   3. *Legionella pneumophilia* [Saha *et al*, 1985].
   4. *Toxoplasma gondii* [McCabe & Remington, 1986].
   5. *Trypanosoma cruzi* [McCabe & Mullins, 1990].

ii) CGD patients have microbiostatic effect on Chlamydia, [Yung *et al*, 1982].

iii) MAI infections have not been so far reported in CGD patients.

iv) Several patients with CGD (sex-linked as well as non-sex-linked) have been reported to live reasonably normal lives.

Direct evidence for O_2-independent mechanisms:-

i) Phagocytosis of *M. bovis* by murine bone marrow macrophages activated by IFN-γ failed to induce oxidative burst [Kaufmann, 1988c].

ii) Scavengers of ROI, including superoxide dismutase, catalase, histidine etc. failed to reverse the growth inhibition of *M. bovis* caused by IFN-γ activated murine bone marrow macrophages [Kaufmann, 1988c].

iii) Growth inhibition of *M. bovis* is induced in unstimulated murine bone marrow macrophages by chemicals i.e. chloroquine, tetracaine and tributylamine, that enhance phagosome-lysosome fusion [Kaufmann, 1988c]

iv) Intracellular killing of virulent *M. tuberculosis* is mediated by reactive nitrogen intermediates in activated mouse macrophages [Chan *et al*, 1992].

There is controversy in the literature about the expression and activity of RNI pathway in human macrophages. Indirect evidence for the presence of this mechanism in humans is as follows:

i) Human neutrophils produce nitric oxide [Salvemini *et al*, 1989].

ii) Inorganic nitrogen oxides found in circulation are excreted under septic conditions following macrophage activation [Petros *et al*, 1991].

iii) Endothelium-denuded human blood vessels treated with TNF and IFN-γ have been shown to have enzymatic activity dependent on arginine, NADPH and BH4 [Mulsch *et al*, 1991].

iv) It has been shown that cytokine-stimulated human monocyte-macrophages inhibit microbial growth *in vitro* through an L-arginine NO-dependent pathway [Cammeron *et al*, 1990, Summersgill *et al*, 1991].

v) Freshly isolated human hepatocytes secrete elevated levels of cyclic GMP and nanomolar amounts of NO_2^-/NO_3^- in response to a cytokine cocktail, and addition of NGMMLA reduces the production of both [Nussler *et al*, 1992].

Apart from the indirect evidences of the presence of nitric oxide pathway in human macrophages a controversial report claims that human macrophages derived from blood monocytes and stimulated with TNF and granulocyte-macrophage-stimulating factor (GM-CSF) utilise a reactive nitrogen intermediates in the killing of intracellular *M. avium* [Denis, 1991b].

Chan *et al* (1992) showed that murine macrophage cell line and murine peritoneal macrophages activated with gamma interferon lipopolysaccharide or tumour necrosis factor alpha, inhibit and also kill phagocytosed *M.tuberculosis* and this capacity of macrophages does not involve the ROIs. They showed that

the ROI-deficient cell line D9 and the inhibitors of ROIs did not affect the killing of *M. tuberculosis.* Bermudez, (1993) compared the role of nitric oxide in the killing of *Listeria monocytogenes* and *M. avium* by activated human and murine macrophages with γ-IFN, TNF and GM-CSF. Increased production of NO by the murine cells was observed after their activation with these cytokines whereas, there was no increase in the production of NO by human monocyte derived macrophages. Neither competitive inhibition of arginine by NMA nor removal of arginine by arginase had any effect on the killing of *M. avium* both by murine and human macrophages. On the contrary, killing of *Listeria monocytogenes* was reversed by NMA and arginase in case of murine cells where as they had no effect on its killing in human macrophages.

Excessive amounts of NO being produced in *Salmonella* infections can lead to bystander autotoxicity and result in immunosupression. A provocative link between NO production and natural resistance has been cited [Vidal *et al,* 1993]. Limited structural homology of recent clone product of the Bcg gene with known eukaryotic membrane-transport systems, suggested a macrophage-specific NO-transporter fubction for this novel protein [Vidal *et al,* 1993].

1.5 A RESUME OF EXPERIMENTAL METHODS AND THEIR TECHNICAL ASPECTS

The current literature concerning intracellular mycobactericidal effector functions is shrouded in controversy. Variability and inconsistency of methods used by different laboratories has made it difficult to determine the facts. Subtle variations in the experimental conditions could easily be responsible for the discrepancies reported. Biological parameters, inherent variability of the responsiveness of human macrophages and the virulence of the particular microbial strains are other possible causes of variation. Some of the major technical difficulties and potential artefacts relating to a range of factors are listed and discussed;

1.5.1 Effector cell and its microenvironment.

1.5.2 Bacterium and its microenvironment.

1.5.3 Variation between phagocytes and mycobacterial species.

1.5.4 Techniques of assessing mycobacterial viability.

1.5.1 Effector cell and its microenvironment.

Monocytes which originate in the bone marrow, leave the circulation at random and migrate to tissues and body cavities where they differentiate into macrophages [Van Furth, 1985]. Macrophages 15-20, μm in diameter morphologically different from lymphocytes have an abundant cytoplasm and a relatively small indented nucleus. The cytoplasm contains a well-developed Golgi complex, which is an extensive vacuolar system comprising phagosomes, endosomes, lysosomes and phagolysosomes. The cytoskeletal system contains microfilaments and microtubules. The plasma membrane of macrophages bears over 30 known receptors, including the receptors for the Fc portion of IgG and the third component of complement (C3). This ability of macrophages to bind to the Fc of IgG and to C3b is important for ingestion of antibody and complement-coated micro-organisms, called immune phagocytosis [Zuckerman *et al,* 1979]. A role of complement was also implied by looking at differences in uptake of mycobacteria by human monocytes [Schwartz *et al,* 1988]. Macrophages in tissues and body cavities exhibit a great diversity of phenotypic and functional characteristics. They are long lived phagocytic cells with a life span of the order of months under steady state conditions [Van Furth, 1985]. Mackaness used the mouse model of Listerial infection to elucidate the relationship between CMI and macrophage activation. Mackaness pioneered work on Listeriosis in mice and coined the term "activated macrophages" [Mackaness, 1962, 1964, Blanden *et al,* 1969]. Macrophages from immunised animals had greater bactericidal properties than those from non-immunised animals [Lurie, 1942].

Macrophage activation involves processing of whole bacteria, antigen presentation to T helper lymphocytes and induction of lymphokine secretion [Douvas *et al,* 1985]. Activated macrophages have a greater spreading activity on glass, are larger, have more hydrolytic enzymes, increased expression of Class II antigen and exhibit an increased oxidative metabolism meaning increased O_2- consumption, H_2O_2 and O_{2-} production. They have greater pinocytotic and microbicidal activity, and an enhanced tumoristatic or tumoricidal activity than normal macrophages [Van Furth, 1985]. Macrophages scavenge and destroy bacteria when activated. Macrophage activation is a local phenomenon restricted to the inflammatory lesion and is particularly marked within granulomata [Dannenberg *et al,* 1968, 1972], and is a stepwise chain of

events in which cells in different stages of maturation and/or differentiation become receptive to primary activating signals to acquire cytostatic or cytotoxic effector responses.

The macrophages employ both oxygen dependent and independent mechanism in killing the invading organism. The study of human cellular immunity usually depends on the blood as the source of monocytes. There are potential differences between circulating monocytes and tissue macrophages. The differentiation of monocytes to macrophages *in vitro* involves a gradual transition of morphologic, physiologic, and biochemical changes, which depend on the method of isolation used.

The following characteristics of monocytes develop over time, as they differentiate to macrophages [Mussons *et al,* 1980, Leslie *et al,* 1982, Sandhaus *et al,* 1982].

1. Increased stimulated release of lysosomal enzymes.
2. Increased and then decreased 5'-nucleotidase activity.
3. Increased spontaneous lysozyme secretion.
4. Increased size and protein per cell.
5. Increased and then decreased superoxide production.
6. Increased growth factor production.
7. Changes in elastase activity from a neutrophil-like enzyme to a macrophage-like enzyme.
8. Increased number of lipid inclusions.

The use of monocytes as models of macrophage anti-mycobacterial effector function may therefore have little relevance to the *in vivo* situation. The use of *in vitro* cultured monocyte-derived macrophages however also have potential inherent technical problems. For example, variability is introduced by the use of various periods of pre-culture of monocytes. Monocytes partially purified by centrifugation, adhere readily to glass or plastic surfaces and show normal differentiation to typical macrophages after 5-7 days in culture [Boyum, 1983, Johnson *et al,* 1977, Zuckerman *et al,* 1979]. It was found that monocytes develop morphologic and biochemical characteristics of macrophages by 14 days [Rinehart *et al,* 1979]. It has been shown that fresh monocytes are fully capable of undergoing intense respiratory burst once effectively triggered. However *in*

vitro-derived macrophages show little oxidative activity but the oxidative killing capacity in *in vitro*-derived macrophages can be restored by activated T cells [Rinehart *et al,* 1979].

Human peripheral blood monocytes cultured *in vitro* in the presence of autologous serum differentiate into cells that resemble tissue macrophages morphologically, biochemically, and functionally [Hammerstorm, 1979]. Conditions of culture and serum supplementation are likely to have a major bearing on the effector functions of monocyte-derived macrophages [Crowle & Poche, 1989]. Macrophages mature differently in low serum concentrations [Nakagawara *et al,* 1981, Musson, 1983]. Some researchers found the use of fresh monocytes, or cells precultured for no more than 2-3 days, preferable because with time the cells become increasingly permissible to intracellular growth of mycobacteria, while others thought that 7 day old monocyte-derived macrophages consistently demonstrate greater phagocytosis than do freshly adherent monocytes [Ellner, 1990].

Serum supplements also make a difference, 10% human serum makes macrophages circular and increases the amount of lipid inclusions [Musson, 1983]. Human monocytes cultured for 7 days in medium containing 5% autologous serum increase their intracellular levels of the lysosomal enzymes, beta glucosaminidase, beta glucoronidase and acid phosphatase, which is dependent upon the concentration of serum that is present during the 7 days in culture period.

The percentage of the initial adherent monocytes and macrophages is yet another variable. The adherent cells present after 7 days in culture vary from donor to donor [Mussons, 1983]. Monocytes isolated by plastic adherence yield better results, as compared to any other method of separation of monocytes [Zerlauth *et al,* 1991]. 4-day old monocyte-derived macrophages were preferred because careless handling of fresh monocytes can activate the cells which have an impaired response to further stimulation [Bermudez & Young, 1989]. Cyclic AMP has been found to promote differentiation in a macrophage cell line thus one might expect cAMP levels to increase during monocyte differentiation. On the other hand, the cytotoxic capacity of monocytes increases dramatically during monocyte-macrophage differentiation, but this function is inhibited by cAMP [O' Dorisio *et al,* 1984, Rinehart *et al,* 1979].

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1.5.2 Bacteria and its microenvironment.

Cell/mycobacterium interaction depends upon the species, strain, and phenotype of the mycobacterium, as well as on the macrophage and its environment. There are some conflicting data on whether intracellular pathogens trigger release of ROS, since factors like age, type, and source of phagocytes, species, strains of organism used, and uptake of the organism in the assay, could all effect the results, [Wilson *et al*, 1980, Brett & Butler, 1988]. Differential susceptibility towards different cell types among different strains of *M. tuberculosis* among other factors, could explain the discrepancy in the findings of different observers [Rook *et al*, 1986a, Douvas *et al*, 1985]. Different strains of *M. tuberculosis* were found to be differently susceptible to the growth inhibiting effects of IFN–γ, [Flesch. *et al*, 1987]. It is difficult to establish that 100% of the test inoculum is taken into macrophages as opposed to surface association during the long culture periods involved, such that some workers have used centrifugation to obtain satisfactory intracellular infection [Stokes & Collins, 1988]. Macrophages usually die when they take up more than 5 to 10 mycobacteria per cell *in vitro* [Filley & Rook, 1991]. A bacteria to cell infection ratio of 1:1 was found to be the optimum to result in almost all the bacteria being taken up inside the cells [Blanchard *et al*, 1991, Stach *et al*, 1984].

The use of serum is yet another source of variability, 10% human serum has been found to be inhibitory to mycobacterial growth but there have been conflicting reports that human monocytes in 20% human serum inhibited BCG. On the other hand serum has been reported to enhance the growth of *M. tuberculosis* [Rook, 1990a]. Others have reported that *M. avium* grows in 1% serum, and is inhibited in 5-50% human serum [Crowle, 1990]. The use of 5% serum was reported to inhibit the growth of *M. avium*, where a combination of cytokines was used [Denis, 1991d]. Iron supplements are also essential for mycobacterial growth and human serum is effectively iron-deficient for mycobacteria [Kochan, 1973].

The antibiotics used in culture medium also have growth-inhibiting effects, especially in bacterial killing experiments [Cole & Brastoff, 1975, Crowle *et al*, 1984]. Human macrophages when activated with TNF-α were found to kill *M. avium in vitro* [Bermudez & Young, 1988] but a very high concentration of penicillin (1000U/ml) in the culture medium could account for this effect. Others have also found that 50 or 100 U/ml

of penicillin within culture medium is inhibitory to *M. avium* growth [Crowle *et al,* 1984, Crowle, 1990]. The mechanism of this inhibitory effect has been proposed to be due to high intracellular levels of penicillin within macrophages sufficient to retard bacterial growth [Cole & Brastoff, 1975].

1.5.3 Variation between phagocytes and mycobacterial species.

Uptake of bacilli by phagocytic cells and subsequent killing varies from donor to donor. Macrophages of blacks have been reported to have a higher bacterial uptake potential, and killing ability than their white counterparts, but not in their monocytes [Crowle & Elkins, 1990]. The rate of development of infection, once established, depends largely on the relative permissiveness of successfully-infected cells, and this permissiveness is greater in cells from black donors than in cells from white donors. This permissiveness was attributed to the presence of autologous serum in the culture medium [Crowle & Elkins, 1990].

Variation of host resistance between different animal species is thought to be a very important factor in susceptibility to mycobacterial infection. Various animal models including mice, rabbits, and guinea pigs have been employed, to study the disease. A lot of progress has been made in elaborating mycobacterial immunity in these animals. Human research however, due to the obvious technical and ethical difficulties, involved in working on patients, has not kept pace. *In vitro* and *in vivo* effects in animal model systems, moreover cannot be taken as a true picture of what may occur either in the *in vitro* or *in vivo* milieu in humans.

1.5.4 Techniques of assessing mycobacterial viability.

Colony forming unit (CFU) assays are less reliable for those strains of mycobacterium which show extensive clumping [Ellner, 1990]. Mycobacteria have to be sonicated to disrupt clumps; which has been found not to effect bacterial viability, whereas vortexing was associated with about 50% loss of the original inoculum, which could be misinterpreted as bacterial killing [Denis, 1991c]. A detailed description of the techniques of assessing mycobacterial viability are given in detail in section VIII.

The following measures were taken in this study to reduce the technical problems detailed above;

1) The same macrophage donors were tested in the experiments for repeatability.

2) The percentage of adherent infected/un-infected cells was determined at each time point of each experiment and for each donor tested.

3) CFU and radiolabelling assays were performed in parallel.

4) No antibiotics were used in the cell cultures.

5) The presence of extracellular organisms was minimised by careful and repeated washing of the cultures after initial phagocytosis.

6) Three minutes of sonication and 0.2% saponin (final concentration) as detergent were used to minimise clumping errors.

7) 4-day old monocyte-derived macrophages were regularly used as the effector cells in the macrophage experiments. These were cultured in 10% and 1% autologous serum for BCG and MAI strains respectively.

8) The same strains of mycobacteria, and the same bacterial stock cultures were used in all the experiments.

1.6 AIMS OF THE PROJECT

1) To develop and standardise methods to determine intracellular mycobacterial growth/viability within phagocytic cells.

2) To investigate the role of neutrophils in human anti-mycobacterial immunity.

3) To investigate the role of recombinant cytokines in human anti-mycobacterial immunity.

4) To investigate the involvement of reactive oxygen intermediates (ROI) and nitric oxide intermediates (RNI) in human and murine anti-mycobacterial immunity.

5) To investigate the role of cytolysis caused by CD4+ T-cell mediated cytolysis of infected human macrophages on mycobacterial survival.

6) To study TNF-α production by mononuclear cells and intracellular mycobacterial growth rates within these cells from multicase TB families.

CHAPTER 2

Techniques of assessing mycobacterial growth/viability and their application in determining intracellular mycobacterial growth within human monocyte-derived macrophages

2.1 Introduction

Research on mycobacterial infections is being held back by lack of suitable, accurate and rapid methods of determining bacterial growth and viability. In this initial phase of the study currently available radiometric and colony counting methods were assessed for their accuracy and reproducibility in determining mycobacterial growth. These included; [^{3}H] uracil, [^{3}H] uridine, and [^{3}H] glycerol incorporation, for radiolabelling BCG/MAI. A new technique, "microcolony counting" in which BCG colonies are grown in Middlebrook 7H9 broth, was also evaluated against standard agar culture methods. These assays were then applied to determine mycobacterial growth/viability following release of bacteria from human monocyte-derived macrophage cultures.

2.2 Materials & Methods

2.2.1 Preparation of Bacillus Calmette-Guerin (BCG) and *Mycobacterium avium intracellulare* (MAI) stocks

BCG and MAI stock solutions were grown in Middlebrook 7H9 broth (Difco, Detroit, Michigan, USA) supplemented with 10% Middlebrook ADC enrichment media (Difco, Detroit, USA) and 0.02% Tween 80 (Sigma), for 1 week at 37°C/5% CO_2. The bacterial suspensions were centrifuged at 6000rpm for 20 minutes to remove the 7H9 medium, and resuspended in RPMI-1640. These were then sonicated in a sonicating waterbath (Heathkit) for 3 minutes to disperse clumps and dispensed into 2ml aliquots in sterile plastic tubes and stored at -70°C, (which does not alter the bacterial viability). The bacterial concentration in colony forming units (CFU) of the stock BCG was then determined by microcolony counting (section 2.2.5) and the stock MAI by agar plate counting (section 2.2.6).

2.2.2 Preparation of human monocyte-derived macrophage monolayers

PBMC were routinely isolated from 50ml samples of defibrinated venous blood taken from normal human donors. The blood was centrifuged for 10 minutes at 400g (MSE, Mistral 2000) to allow separation and removal of the serum. The buffy coat fraction was carefully removed and resuspended to twice the original volume with RPMI-1640 (Gibco, Paisley, Scotland) supplemented with 1% (150 mM) glutamine (Sigma, St Louis, USA). This was layered over Ficoll-paque, density 1.077+/- 0.001 g/ml (Pharmacia, Uppsala, Sweden) and centrifuged at 400g for 25 minutes. The cells from the interface were then washed twice in RPMI-1640 and adjusted to 2.5 x10^6/ml in antibiotic-free RPMI-1640 +10% autologous human serum. The cells were plated out in 96-well, flat-bottomed microtitre tissue culture plates (Flow laboratories, England) in 200µl aliquots/well. Following overnight incubation at 37°C/5% CO_2, the plates were gently washed by resuspending in the same medium (x 5) and removing the supernatant medium containing the non–

adherent cells and 200μl aliquots/well of fresh medium was then added. At this stage the adherent cells were referred to as one day-old monocytes in subsequent experiment protocols. Adherence studies were performed initially on the monocyte cultures at this stage to determine the percentage adherence of the PBMC following overnight incubation (as described in section 2.2.3). The adherent monocyte-enriched cultures were then incubated for four days at 37°C/5% CO_2 to allow differentiation into macrophages. The cells were then examined by inverted phase contrast microscopy (Olympus, CK2, Japan) to confirm that the macrophages were confluent.

2.2.3 Survival of adherent human monocyte-derived macrophages in plastic tissue culture plates.

PBMC were separated as detailed in section 2.2.2. The adherent monolayers were then cultured at 37°C/5% CO_2 for various lengths of time. Adherence studies were then performed on the monocyte cultures incubated with and without BCG at an infection ratio of 5BCG/cell. Infection of monolayers was carried out as detailed in section 2.2.4. The adherent infected and un-infected monolayers were then scraped off with a plastic tip, and a viability count was performed on 1:1 dilution of the cell suspension with 1% gentian violet in acetic acid in the representative wells. The nuclei of adherent cells were then counted in a counting chamber. Similarly, the counts were performed on day 1, 2, 4, and 7 days post cultured cells.

2.2.4 Infection of human monocyte-derived macrophage monolayers

A BCG stock aliquot was thawed, sonicated in a sonicating waterbath (Heathkit) for 3 minutes to disrupt the BCG clumps without affecting bacterial viability and adjusted to the required infection concentration in RPMI-1640 +10% autologous human serum. The bacteria were then incubated for half an hour at 37°C/5% CO_2 to allow opsonisation and then added to the cell cultures in 20μl aliquots/well. The plates were then incubated for 4 hours to allow phagocytosis to occur, and the non-phagocytosed bacteria were removed by resuspending them in their own medium (x 5), which was then discarded. The infected cultures were then washed with fresh warm medium by pipetting up and down (x 5) and discarding the supernatant. 100μl aliquots/well of fresh warm medium were then added and sample wells were harvested at different time

intervals post-infection, by lysis of the adherent macrophage monolayers with 20μl of 2% saponin. The well contents were then transferred to 1 ml sterile Eppendorf tubes (Appleton woods, Birmingham, U.K) and stored at -70°C before estimating bacterial viability by the microcolony counting method (section 2.2.5).

2.2.5 The microcolony counting method for determining viability of BCG

BCG aliquots were thawed and sonicated for three minutes in a sonicating waterbath (Heathkit) to disrupt the BCG clumps without affecting bacterial viability. Aliquots of 50μl were then added in triplicate to the first wells of a 96 well flat-bottomed plate containing 200μl of Middlebrook 7H9 broth + 0.2% glycerol + 10% ADC supplement per well. The contents of the wells were mixed thoroughly by repeated pipetting (x 20) and then serial 1:5 dilutions were made by the repeated transfer of 50μl aliquots to the next well in the row. The last 50μl of the suspension was discarded. The plates were then wrapped in cling film. This permits gas exchange whilst reducing media evaporation, prevents fungal contamination, and permits microscopical examination without removal of the film. The plates were incubated at 37°C/5% CO_2 for 14 days to achieve optimum development of countable colony forming units (CFU). The wells containing (20-40 CFU/well) were then counted using an inverted microscope (Olympus CK2, Japan) at (x1000) magnification (Figure 2).

Calculation of colony forming units (CFU)

CFU/ml	=	Av x Dil x 20
Av	=	Average of counts in replicate wells.
Dil	=	Dilution factor of the well.
x 20	=	(CFU/ml) Correction factor.

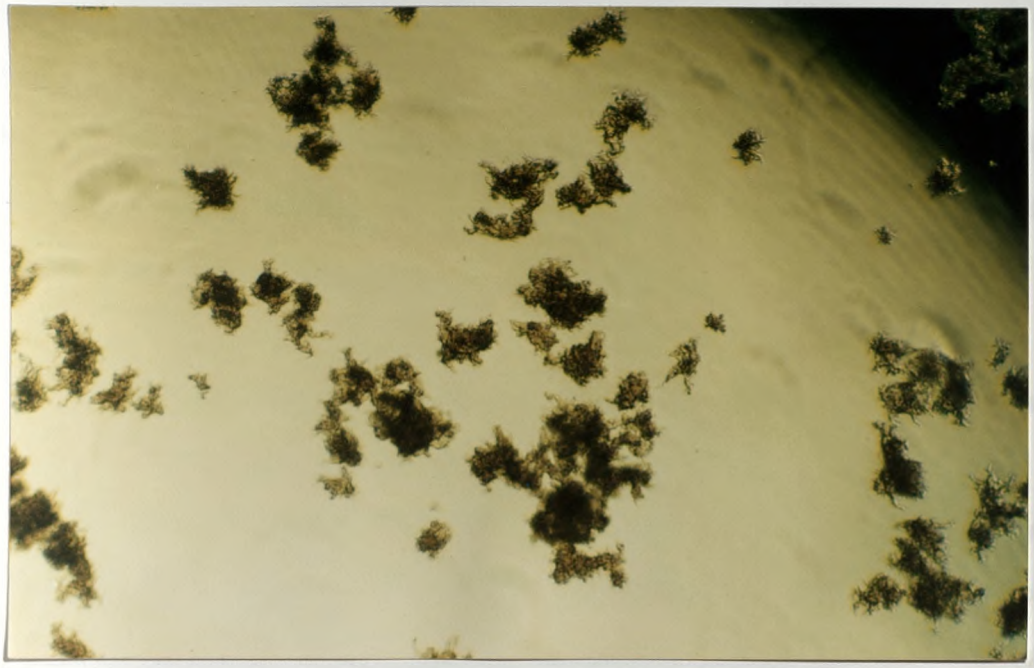


Figure 2

BCG microcolonies under inverted microscope (x1000) in 96-well flat bottomed tissue culture plate.

2.2.6 Agar plate counting for determining viability of *Mycobacterium avium intracellulare* (MAI).

MAI samples of unknown concentrations were plated out on 7H10 Middlebrook agar supplemented with 10% Middlebrook OADC (Difco, Detroit, USA). The bacterial suspension was then sonicated for 3 minutes in a sonicator waterbath and then three different dilutions (1:10, 1:100. 1:1000) were prepared in 7H9 broth in a 96-well flat bottomed tissue culture plate. 10μl aliquots were plated per dilution on 60mm petri dish (Flow, England.). The plates were then wrapped in cling film and incubated at 37°C/5% CO_2 for 14 days to obtain visible bacterial colonies. The results were expressed in mean number of CFU ± SD. All MAI work was carried out under strict supervision in a Category III cabinet.

2.2.7 Radiolabelling assays for determining growth of BCG and MAI in 7H9 broth culture.

Stock aliquots of BCG and MAI, of known concentration were thawed and sonicated for 3 minutes in a sonicating waterbath to disrupt clumps. Bacterial concentrations within a range 10^2-10^8 bacteria/well in quadruplicate, were prepared in 96-well flat bottomed tissue culture plates containing 100μl of 7H9 Middlebrook broth. The following radioactive isotopes were then prepared in Middlebrook 7H9 broth and added to the bacterial cultures in 20μl aliquots/well.

a) (5,6-^{3}H) uridine, 10mCi/ml, specific activity; 38Ci/mmole (ICN Flow. U.K)

b) (6-^{3}H) uracil, 5-10mCi/ml, specific activity; 28 Ci/mmole (Amersham International plc, Amersham, U.K)

c) (2-^{3}H) glycerol, 10mCi/ml, specific activity; 15mCi/mmol. (Du-pont, U.K Limited)

Note:- No supplemental glycerol was added to the basic medium to avoid labelling of glycerol.

The plates were then incubated at 37°C/5% CO_2 for various periods of time (4 hours to 7 day post-incorporation) to allow incorporation of the radiolabel into the proliferating bacteria. The bacterial cultures were then assessed microscopically for growth and contamination. The cultures were then harvested with distilled water onto glass fibre filter papers (Printed Filtermat A, 1205-401, Pharmacia Wallac U.K) using an automated cell harvester (Skatron Combi CH, Finland). The filter mats were air-dried and counted for 60 seconds i.e. counts per minute (cpm), in a liquid scintillation counter (1205 Beta plate™, Wallac, Pharmacia, U.K) .

2.3 Results

2.3.1 The microcolony counting method for determining viability of BCG

Initial studies were performed to determine the reproducibility of the microcolony counting method as a technique for determining BCG viability. Separate experiments were performed using a new method of counting BCG in Middlebrook 7H9 broth, referred to as the "Microcolony counting method". BCG cultures were grown in 7H9 medium in the absence of Tween to promote formation of discernible microcolonies by 14 days post-incubation. Briefly, 50μl aliquots of disrupted macrophage lysates were serially diluted (1 in 5) in triplicate in 200μl liquid Middlebrook 7H9 broth (Difco, Detriot, USA) + 0.2% glycerol + 10% ADC (Difco, detroit, USA) supplement in 96-well flat-bottomed microtitre plates. The plates were then incubated at 37°C/5% CO_2 and individual discrete colonies were counted by inverted microscopy (Olympus CK2, Japan). Following assessment of microcolonies over 10-20 days it was found that day 14 post-incubation was the optimum sampling period (Table 2.1), as by this time clearly discernible microcolonies of optimum size had developed for easy counting under an inverted microscope (x1000 magnification). Later time points resulted in crowding of microcolonies, due to overgrowth, however the total number of colonies did not increase beyond day 14 (Table 2.1). A high level of reproducibility was thus obtained within these experiments by this technique.

A comparative study was also made to determine the growth of BCG within 7H9 broth as compared to either 7H10 or Dubos-Oleic agar. BCG growth, as determined by colony forming unit's (CFU) estimation, was found to be similar in 7H9 culture broth as in either of the standard agar cultures (Table 2.2). CFU estimation on a range of BCG concentrations was also determined by both 7H9 broth culture (microcolony counting) and the standard method of colony counting on 7H10 agar. Table 2.2 shows the similarity of CFU's obtained for two such bacterial dilutions by both methods.

Table 2.1

Effect of incubation time on the number of colony forming unit's (CFU) determined by the broth microcolony counting technique

Incubation time (days)			10	14	18
Well no in row[a]	**Final volume[b]**	**Number of experiments**	**Mean+/- SD**	**Mean+/- SD**	**Mean+/- SD**
5	200µl	14	30+/-7	64+/-13	Not countable[c]
5	100µl	27	23+/-3	50+/-8	Not countable[c]
6	200µl	14	6+/-1	12+/-3	12+/-3
6	100µl	26	4+/-0.5	10+/-3	10+/-3

[a] Samples taken from successive row at different descending bacterial seeding concentration in 96- well plate.

[b] Volume of bacterial suspension per well.

[c] The microcolonies enlarged in size and diameter merging into each other making it difficult to distinguish one from other.

Table 2.2

Comparison of different culture media in determining growth/viability of BCG

Culture medium	Sample volume[a]	Number of experiments	Number of colonies Mean +/- SD
7H10 agar	200μl[b]	12	57+/-11
Dubos oleic agar	200μl[b]	12	56+/-10
7H9 broth[c]	200μl	27	58+/-9
7H10 agar	100μl[b]	12	14+/-2
Dubos oleic agar	100μl[b]	12	12+/-1
7H9 broth	100μl	27	11+/-3

[a] Total volume of bacterial suspension. Two different sample volumes were tested for comparison.
[b]20μl of the total initial sample was plated onto agar plates, and from that the number of colonies were adjusted for 200μl for comparison with broth culture.
[c]BCG count in 7H9 broth was determined by the microcolony counting method in 96-well plate.

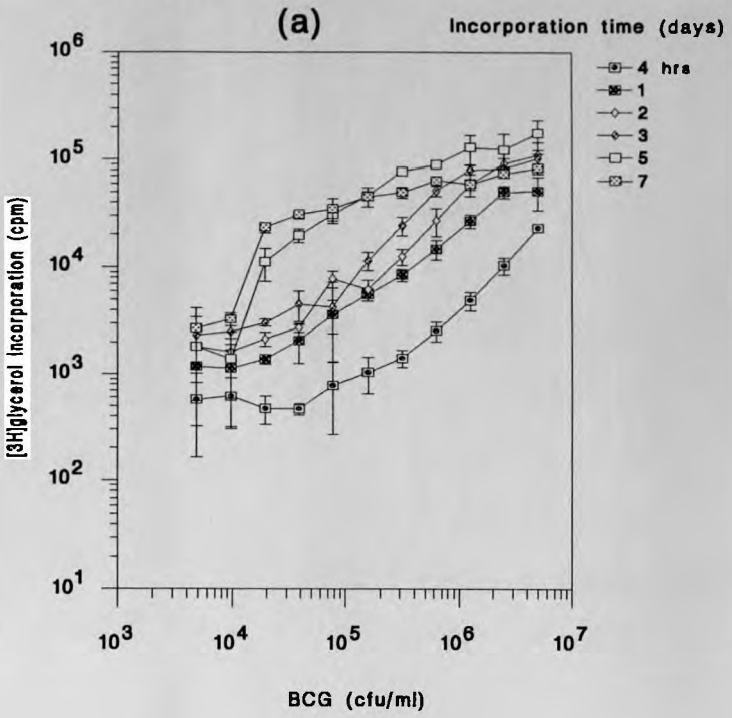
2.3.2 Radiolabelling assays for determining BCG/MAI growth in macrophage-free and macrophage-treated systems.

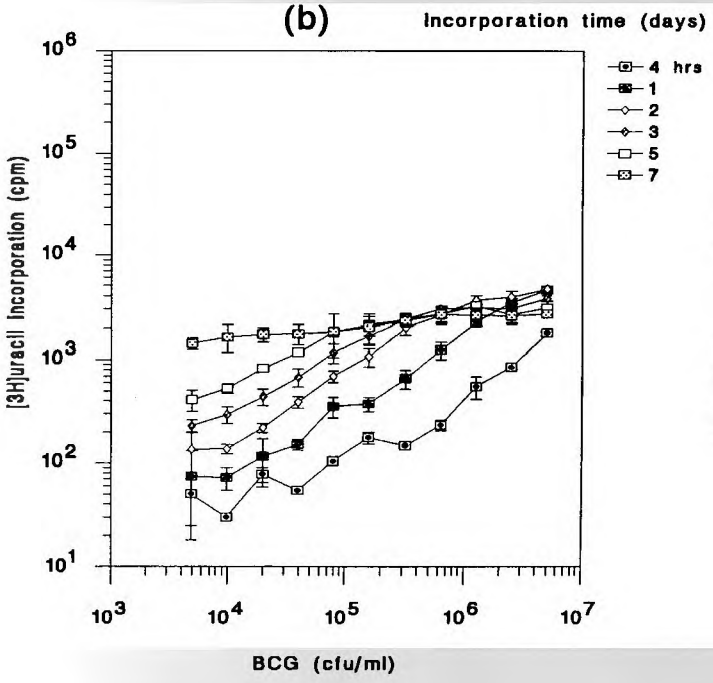
All the available radiolabelling methods of determining mycobacterial viability including; [^{3}H] uracil, [^{3}H] uridine, and [^{3}H] glycerol, were comparatively assessed using BCG/MAI as the target organisms. Bacterial dilutions of 10^2-10^8 bacteria/well were plated in quadruplicate in 7H9 medium in 96-well plates. The radiolabels were added to the growing cultures of BCG/MAI, and a time course of between 4 hours to day 7 post-addition of radio-isotopes was followed. At each time point post-incorporation, the bacterial cultures were harvested and the radioactivity determined as mentioned in section 2.2.7.

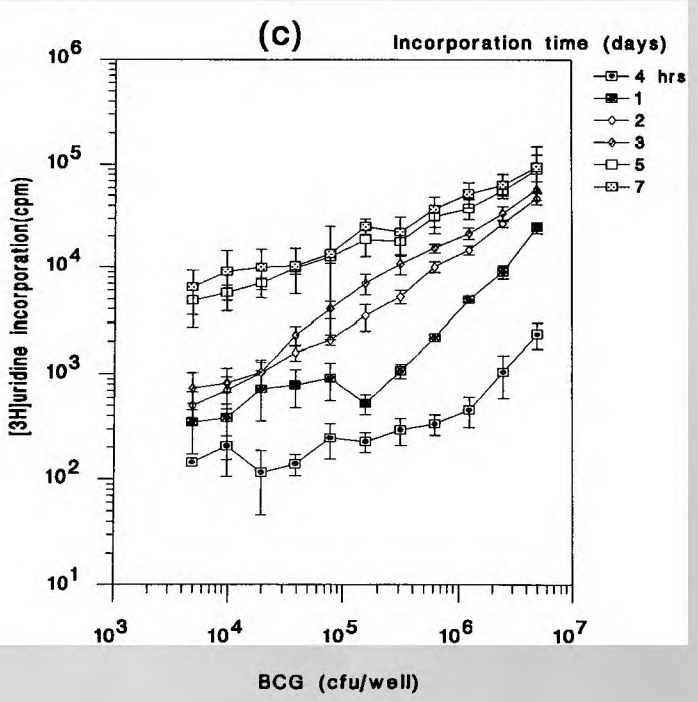
All radio-isotopes were found to be capable of determining BCG viability to varying degrees in a reproducible manner. [^{3}H] uracil, [^{3}H] uridine, and [^{3}H] glycerol started to be incorporated within 4 hours of initiating BCG growth in 7H9 medium (Fig:2.1a-c). A log linear correlation was established for BCG within 24 hours in case of [^{3}H] uracil (Fig: 2.1b) and [^{3}H] glycerol (Fig: 2.1a) and within 48 hours for [^{3}H] uridine incorporation (Fig: 2.1c). Increasing the time of incorporation beyond 48 hours reduced the sensitivity of the assay with each radio-isotope tested. After day 5 post-incorporation, a plateau was reached for [^{3}H] uracil and [^{3}H] uridine, and at this time the assay could not discriminate between different numbers of BCG. In case of [^{3}H] glycerol, increasing the BCG number to more than 10^7 bacilli/well actually reduced the amount of radioactivity detected. The sensitivity ranges of the different radio-isotopes for BCG and MAI with optimum radio-isotope incorporation periods are summarised in Table 2.3.

Similarly experiments were performed to determine the growth of MAI in 7H9 medium culture broth following labelling of the bacteria with one of the following isotopes, [^{3}H] uridine and [^{3}H] glycerol, as described in section 2.2.7. Figures 2.2 a & b & 2.3 (b) indicate that both of the above mentioned radiolabels were incorporated into MAI within 4 hours of addition of the radio-isotope in 7H9 medium. Log linear correlations were established at different days. With the passage of time the growth curves depicting uptake of radiolabels plateaued, resulting in loss of discrimination between different numbers of MAI (fig:2.2a). When the cultures contained $>10^5$ bacteria per well the radio-isotope incorporation of [^{3}H] glycerol into

Figure 2.1
Radiolabelling BCG with various radio-isotopes in culture broth







Figures 2.1(a-c)

These figures illustrate radiolabelling of BCG in 7H9 culture medium with three different radio-isotopes i.e [^{3}H] uracil (a), [^{3}H] uridine (b), and [^{3}H] glycerol (c) over a time course. These experiments were performed to standardise the optimum time of radiolabelling BCG for each radio-isotope tested. Known concentrations of BCG were plated in the first column (1) of flat bottomed 96-well tissue culture plate, and then serial dilutions were performed along the rows (A-H, 12 in number) to give 12 different bacterial concentrations. The bacterial cultures were then radiolabelled and harvested at each time point i.e 4 hours - to 1, 2, 3, 5, and day 7. Each data point represents mean of quadruplicate samples +/- SD (vertical bars).

Figure 2.2 (a)
Radiolabelling MAI with [3H] glycerol in culture broth

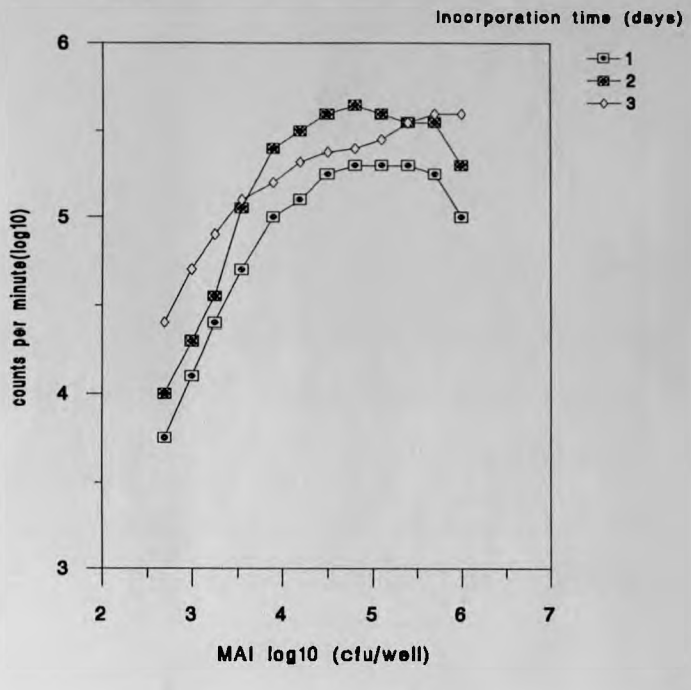
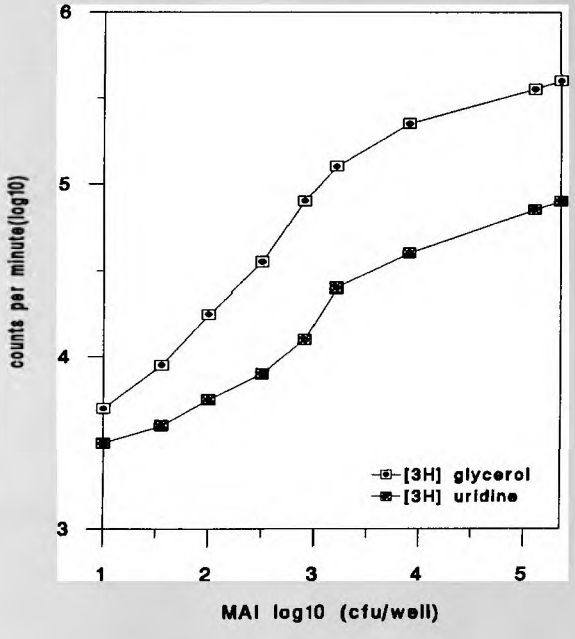


Figure 2.2 (b)
Radiolabelling MAI with various radio-isotopes in culture broth over 3 days



Figures 2.2 (a-b)

These figures illustrate radiolabelling of MAI in 7H9 culture medium with [^{3}H] glycerol over a time course (a), and [^{3}H] uridine and [^{3}H] glycerol over 3 days (b). These experiments were performed to standardise the optimum time of radiolabelling MAI for each radio-isotope tested. Known concentrations of MAI were plated in the first column (1) of flat bottomed 96-well tissue culture plate, and then serial dilutions were performed along the rows (A-H, 12 in number) to give 12 different bacterial concentrations. The bacterial cultures were then radiolabelled and harvested at each time point i.e 4 hours - to 1, 2, 3, 5, and day 7. Each data point represents mean of quadruplicate samples +/- SD < 5% of mean (not shown).

Figure 2.3 (a)
Comparison between radiolabels in labelling BCG in 7H9 culture broth over 24 hrs

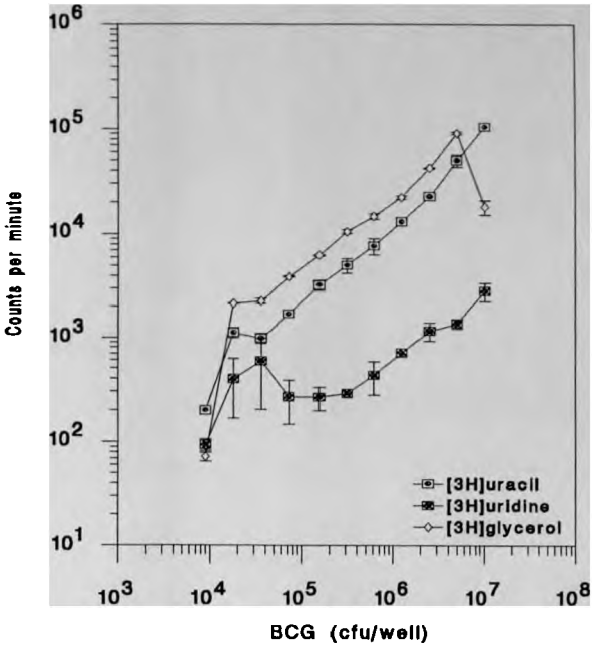
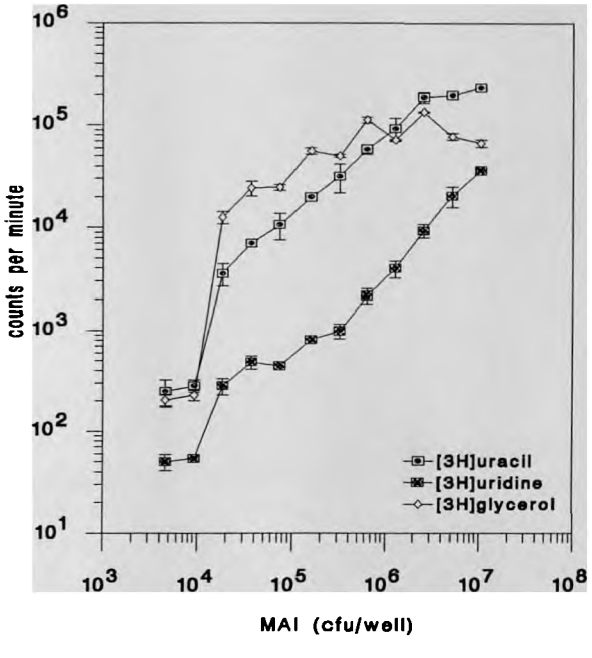


Figure 2.3 (b)
Comparison between radiolabels in labelling MAI in 7H9 medium over 24 hours



Figures 2.3(a-b)

These figures illustrate comparison between different radio-isotopes in labelling BCG (Fig; 2.3a) and MAI (Fig; 2.3b) in 7H9 culture medium. Known concentrations of BCG/MAI were plated in the first column (1) of flat bottomed 96-well tissue culture plate, and then serial dilutions were performed along the rows (A-H, 12 in number) to give 12 different bacterial concentrations. Individual wells were radiolabelled with different radio-isotopes i.e [^{3}H]uracil, [^{3}H]uridine, and [^{3}H]glycerol at the same time. The radiolabelled cultures were harvested at 24hrs post-incorporation. Each data point represents mean of triplicate samples +/- SD.

Table 2.3

Sensitivity ranges of the different radiolabels to determine BCG/MAI concentration in both macrophage-free[a] and macrophage-treated[b] systems

Method	[^{3}H]uracil	[^{3}H]uridine	[^{3}H]glycerol	CFU counting
BCG				
Macrophage-free				
Duration[c] (days)	1	2	1	14[d]
Range[e]	10^4-10^7	$5x10^3$-10^7	10^4-$2x10^6$	200-10^{12} or >
Macrophage-treated				
Duration[c] (days)	3	3	3	14
Range[e]	10^4-10^6	10^4-10^7	10^4-10^6	200-10^{12} or >
MAI				
Macrophage-free				
Duration[c] (days)	1	2	1	14[f]
Range[e]	10^2-10^6	10^2-10^6	10^2-10^6	200-10^{12} or >
Macrophage-treated				
Duration[c] (days)	3	3	3	14
Range[e]	10^2-10^7	10^2-10^7	10^2-10^5	200-10^{12} or >

[a]BCG grown extracellularly (macrophage-free) in 7H9 culture broth supplemented with 10% ADC.
[b]Represents bacteria liberated from 4-day old human macrophages following saponin treatment and then growth/viability determined. In these experiments more than one human donor source of macrophages was used for macrophage-treated systems.
[c]Minimum length of time required for the assay.
[d]CFU counts were determined by the microcolony counting technique as described in Methods 2.2.5.
[e]Sensitivity of the assay to discriminate different numbers of bacterial counts.
[f]CFU counts for MAI were performed on 7H10 agar plates.

MAI actually started declining, whereas [^{3}H] uridine incorporation progressed to estimate > 10^5 bacteria/well.

The results as summarised in Table 2.3 indicate that all the radiolabels could efficiently discriminate between different numbers of bacteria, either which were liberated from cells by saponin treatment, or the ones growing in cell-free cultures. However it was found that [^{3}H] uridine incorporation gave the best correlation between radio-isotope uptake and number of bacteria (fig: 2.1c). It is also sensitive for both BCG and MAI at all the working ranges examined of the following experiments (fig:2.3a &b). It should be noted that in the same experiments it was found that the levels of radio-isotope incorporation for all the radiolabels into the cells alone cultures was negligible i.e. < 10 cpm (results not shown).

2.3.3 Effect of iron supplement on the sensitivity of [^{3}H] uridine radiolabelling assays.

The effect of the addition of ferric ammonium citrate to enhance the sensitivity of the mycobacterial radiolabelling assay method was tested in this series of experiments. The addition of the Iron supplement has been reported to enhance the proliferation of mycobacteria *in vitro*, and hence, may act to increase the sensitivity of the radiolabelling assay. Aliquots of 10^5 bacteria/well (BCG/MAI) were then added to 7H9 broth containing Ferric ammonium citrate solution 50μg/ml (final concentration). The number of proliferating bacteria was determined 3 days post-incubation by radiolabelling with [^{3}H] uridine for 24 hours. Indeed, it was found that addition of iron supplement marginally increased the sensitivity of [^{3}H] uridine incorporation for lower concentrations of MAI (Fig:2.4b) but not for BCG (Fig:2.4a).

2.3.4 BCG and MAI growth in different culture media

These experiments were performed to determine the effect of different media on allowing mycobacterial growth. Aliquots of 10^5 bacteria/well (BCG/MAI) were added to different culture media. The number of proliferating bacteria was determined 3 days post-incubation by radiolabelling with [^{3}H] uridine for 24 hours. The results are represented as counts per minute (cpm) in figures 2.5 a & b. In this assay system RPMI-1640 + 10% human serum was found to be inhibitory to the growth of BCG (Fig:2.5a) and MAI

Figure 2.4 (a)
The effect of iron supplement on radiolabelling BCG

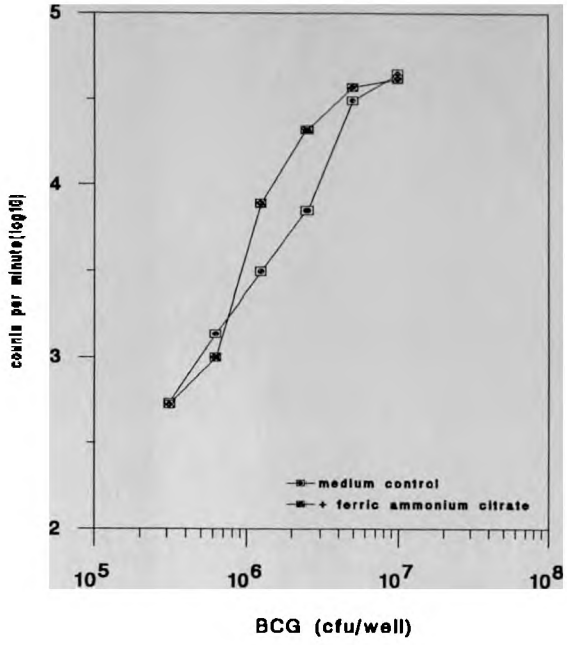
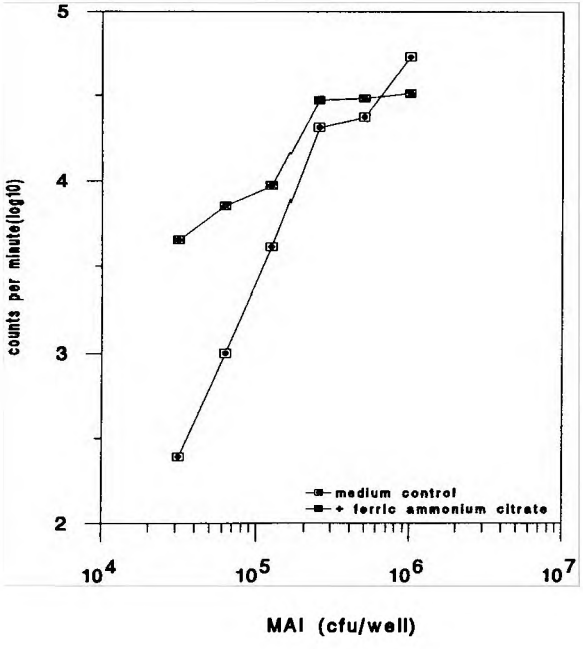


Figure 2.4 (b)
The effect of iron supplement on radiolabelling MAI



Figures 2.4 (a-b)

These figures illustrate the effect of iron supplement on [^{3}H] uridine radiolabelling. Six different concentraions of BCG (a) or MAI (b) were plated out in 96-well plates with iron supplement (Ferric ammonium citrate 50μg/ml). Control bacterial cultures which did not receive iron supplement were also included. 2μCi/well of [^{3}H] uridine was added per well. The bacterial cultures were harvested 48 hours post-incorporation. The results are expressed as $\log_{10}$ values of counts per minute. Each data point represents mean of triplicate, SD of < 5% (not shown).

(Fig:2.5b). Both BCG and MAI also failed to grow in RPMI-1640 medium alone. Middlebrook's 7H9 medium supplemented with 10% ADC was then found to be the optimum media for mycobacterial growth (Fig: 2.5a & b).

2.3.5 Survival of adherent human monocyte-derived macrophages in plastic tissue culture plates.

PBMC were isolated from three normal human volunteers from the University of Birmingham, Medical School, as mentioned in section 2.2.2, and adherence studies on these monolayers were performed as detailed in section 2.2.3. An initial study was performed to determine the percentage loss of adherent cells over the standard time course of the bacterial growth assay (Table 2.4). An initial infection ratio of 5 BCG per macrophage was used, which was found not to be detrimental to the adherent cells. There was a good yield of adherent (i.e. macrophages) from all the donors used in these experiments which were cultured for 4 days, in the presence of 10% fresh autologous human erum. Infection moreover, with BCG was not found to induce loss of adherent cells from the 96-well tissue culture plates. On average 10-15% of the total PBMC plated out, adhered to the plastic plates following overnight (18hrs) culture at $37^{\circ}C/5\%$ CO_2, in both monolayers cultured with, or without BCG infection.

2.3.6 Intracellular growth of BCG within human monocyte-derived macrophages

Monocyte-derived human macrophages were isolated as described in section 2.2.2 from three different donors. Donor 1 (Caucasian female), Donor 2 (Afro-Caribbean female) and donor 3 (Caucasian male). Four days post-culture the monocyte-derived human macrophages were infected with BCG at a bacteria:cell infection ratio of 5:1. Extracellular BCG were removed, and the number of intracellular bacteria determined at 4 hours post-infection as zero-time. The growth of BCG was then followed over 2, 4, and 7 days post-infection. At each time point BCG viability and growth estimates were performed by both microcolony assessment (fig:2.6 a-c) and [^{3}H] uridine incorporation (fig:2.6 a'-c'), respectively, and the growth pattern of BCG within human macrophages for each donor determined. BCG was found to grow intracellularly within monocyte-derived macrophages from all three donors tested. Percentage change in bacterial growth over time course, as assessed by both parameters, was estimated and shown in Table 2.5. All three donors,

Figure 2.5 (a)
BCG growth in different media as assessed by [3H] uridine radiolabelling over 3 days

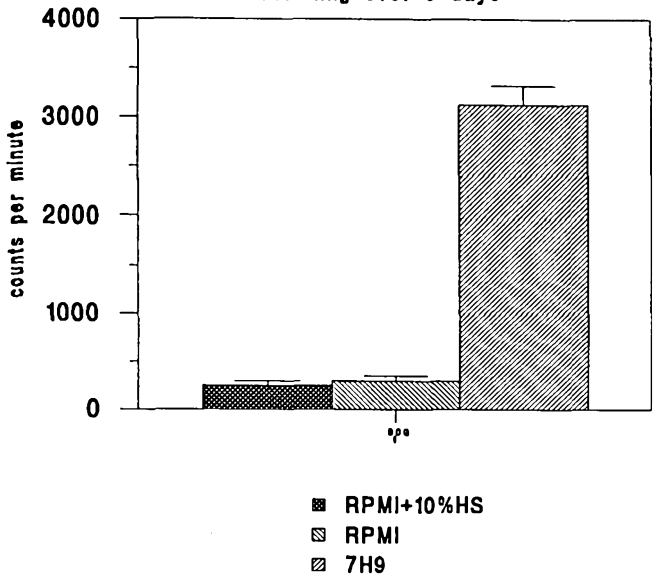
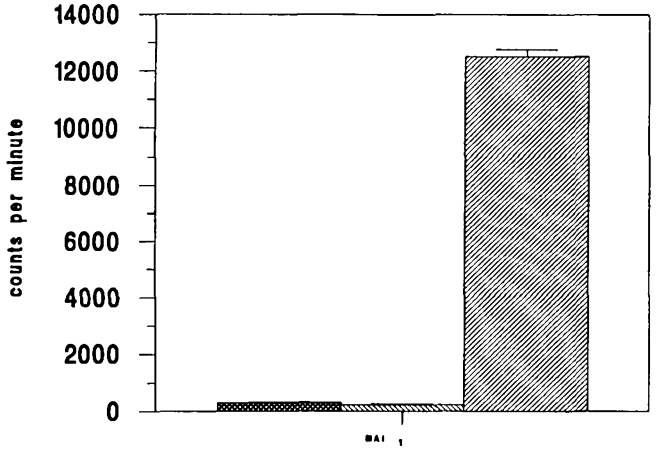


Figure 2.5 (b)
MAI growth in different media as assessed by [3H] uridine radiolabelling over 3 days



Figures 2.5 (a-b)

These figures illustrate the growth of BCG/MAI in different media. Known concentrations of BCG (Fig: 2.5a) or MAI (Fig: 2.5b) were grown in different culture media i.e. RPMI-1640, RPMI-1640 + 10% human serum, and 7H9 medium + 10% ADC supplement. 2μCi/well of [^{3}H]uridine was added per well. The bacterial cultures were harvested 3-days post-incorporation. Each column represents mean of triplicate samples +/- SD.

Table 2.4

Percentage[a] viability of un-infected/infected PBMC remaining adherent to tissue culture plates at various time points post-culture.

Days post-incubation	[b]Mϕ	Perecentage viability		
		Donor 1	Donor 2	Donor 3
0[c]	Un-infected[d]	[f]13+/-2	14+/-3	12+/-0.5
	Infected[e]	12+/-2	14+/-2	12+/-0.5
2	Un-infected	12+/-1	13+/-2	12+/-1
	Infected	12+/-1	13+/-2	12+/-1
4	Un-infected	13+/-2	13+/-1	13+/-1
	Infected	13+/-2	13+/-1	13+/-0.5
7	Un-infected	12+/-1	13+/-2	12+/-1
	Infected	12+/-1	13+/-1	12+/-0.5

[a]Percentage viability is determined by calculating the total number of cells remaining adherent at time post-incubation to the plastic in 96-well tissue culture plates (section 2.2.3). Hence the property of adherence of macrophages was used as an index of their viability.

[b]Macrophages.

[c]Time (0) represents 4 hour post-infection for infected cultures.

[d]Un-infected macrophages were cultured in RPMI-1640+10% autologous serum.

[e]Four-day old human monocyte-derived macrophages were infected with BCG at infection ratio of 5 bacteria/cell.

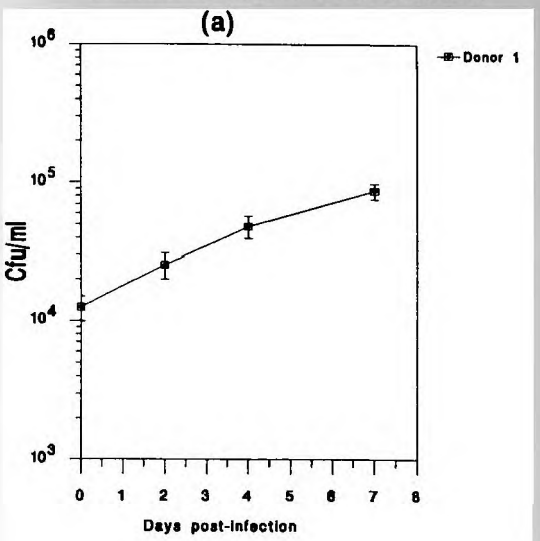
[f]Each figure represent mean of 6 replicates +/-SD. Viability is calculated by the following equation:-
% viability = [Number of adherent cells at time (n)/Total number of PBMC added/well] x 100.
Total number of PBMC added in these experiments were 5 x 10^5 per well.
n = days post-incubation.

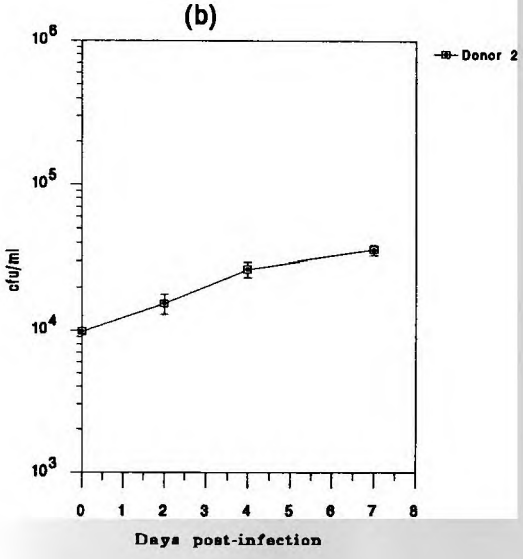
according to both parameters of assessing percentage change in bacterial growth, exhibited good correlation's at day 2 post-infection (+110% to +148% in cpm and +125% to +150% in CFU) estimates. However at day 4 and day 7, such correlations were lost. Indeed the metabolic activity of dividing bacteria at day 7 was maximum, (+638%) by cpm and (+714%) by CFU estimate for donor 3, whereas donors 1 & 2 showed corresponding changes in bacterial growth i.e. +343% to +443% by cpm and +400% to +450% & by CFU respectively (Table 2.5).

Moreover to determine that the patterns of intracellular BCG growth obtained for each donor were consistent, BCG growth curves were obtained for the three donors performed at different days. In these experiments BCG growth/viability was determined by CFU estimate by Microcolony counts as described previously (section 2.2.5). The results, expressed as $\log_{10}$ CFU values for all 3 macrophage donors are illustrated in Figures 2.7a'-c'. The data illustrated, from the 7 different experiments for donors 1 and 2, and the 4 different experiments for donor 3 have been pooled (Table 2.6). For all the macrophage donors at day 2 and day 4 post-infection, the percentage change in bacterial growth is approximately the same i.e. between (+58% to +65%) and (+231 to +298%) respectively. However at day 7, the macrophage cultures from donor 1 and donor 3 exhibited high percentage changes in bacterial growth i.e. +1150% and +1340% respectively, as compared to macrophage cultures from donor 2 which expressed a +712% change in bacterial growth (Table 2.6).

Table 2.6A shows that experiment to experiment coefficient of variation of intracellular BCG growth estimates within a single donor is between 4-12%. In contrast, the coefficient of variation of intracellular BCG growth between the 3 donors studied is < 4%. Hence, potentially, experiment to experiment (i.e. between batch) variation can exceed donor to donor variation, and differences <10% between donors studied on different days needs to be interpreted with caution.

Figure 2.6 (a-c)
Intracellular BCG growth in human monocyte-derived macrophages as determined by microcolony count





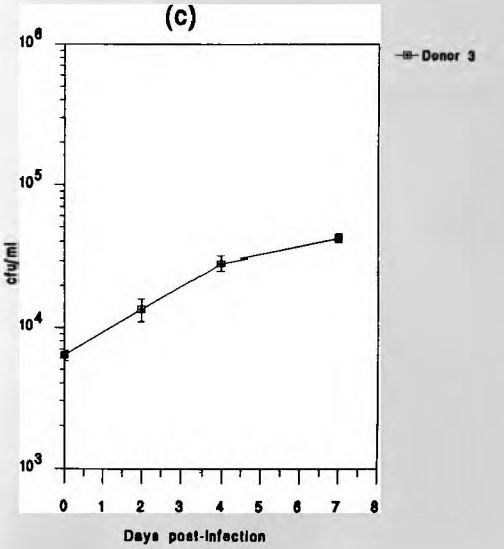
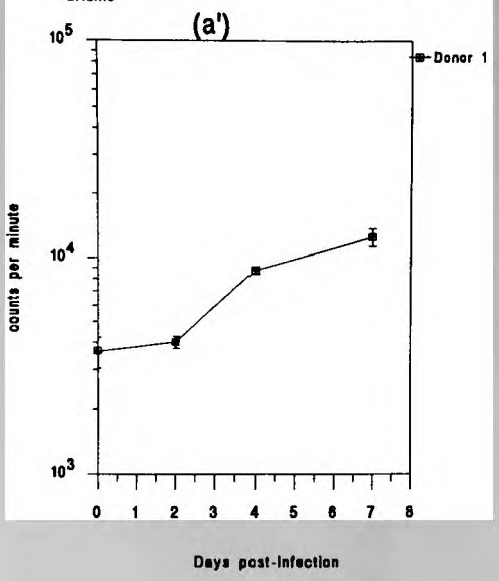
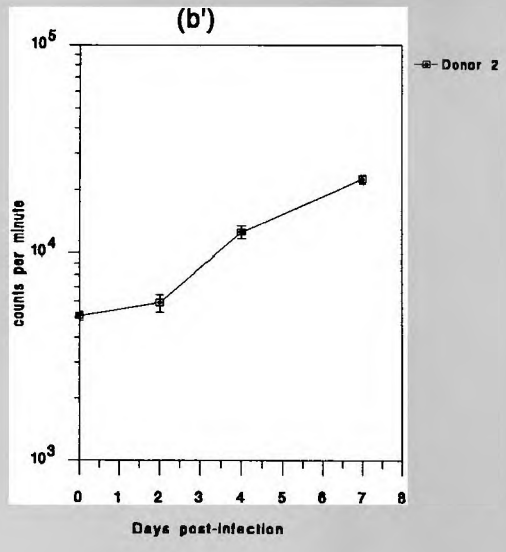
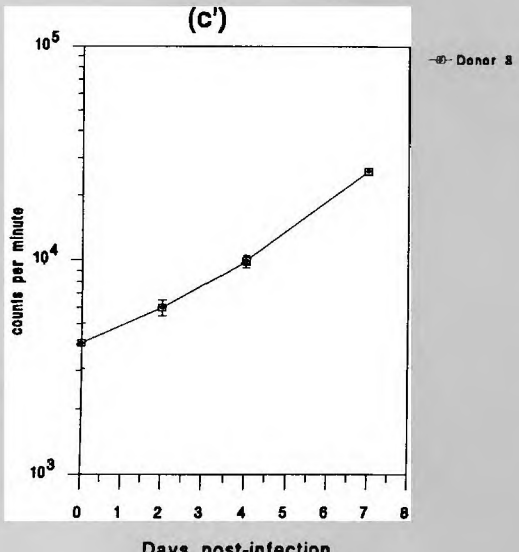


Figure 2.6 (a'-c')
Intracellular BCG growth in human monocyte-derived macrophages as determined by radiolabelling with [3H] uridine







Figures 2.6 (a-c')

These figures illustrate intracellular growth of BCG within 4-day old human monocyte-derived macrophages obtained from Donor 1(a & a'), Donor 2 (b & b') and Donor 3 (c & c'). The growth/viability of BCG was determined by microcolony method (Fig: 2.6 a-c) and is represented as an estimate of CFU/ml (x–axis). The metabolic activity of proliferating BCG was determined by [^{3}H] uridine incorporation (Fig: 2.6 a'-c') and is represented as an estimate of counts per minute (x–axis). Intracellular bacteria were determined 4hrs post-infection as zerotime (0), and 2, 4 and 7 days post-infection (y–axis). Each figure is representative of three experiments performed on different days, where each donor was tested in triplicate at every time point. Each data point is mean of nine replicates +/- SD (vertical bars)

Table 2.5

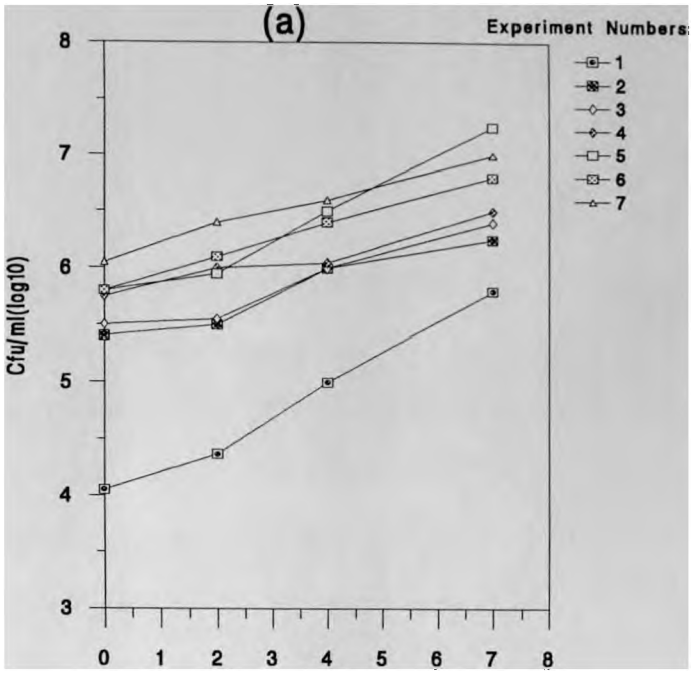
Comparison between [^{3}H] uridine incorporation and Microcolony counting (CFU) in determining BCG intracellular growth within 4-day old human monocyte-derived macrophages

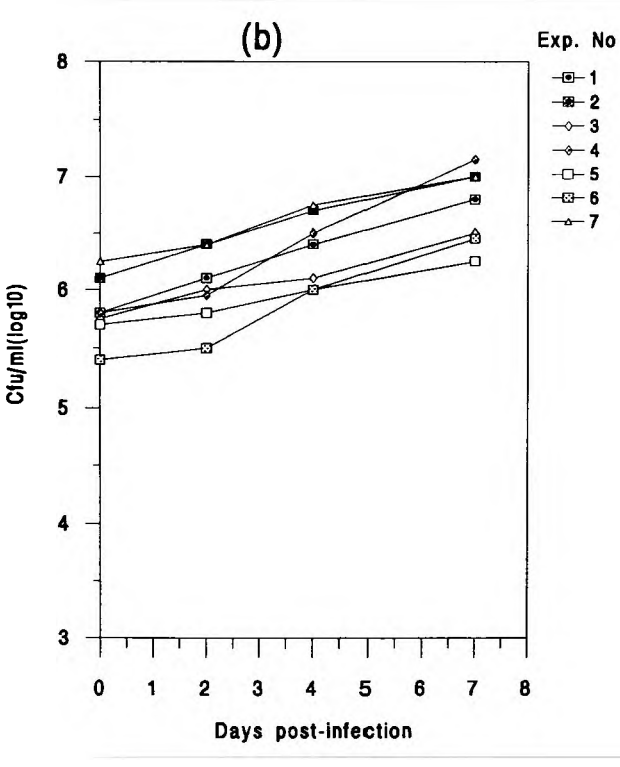
	% Δ[a]					
Days post-infection	**2**		**4**		**7**	
[b]Mϕ donors	**cpm[c]**	**CFU[d]**	**cpm[c]**	**CFU[d]**	**cpm[c]**	**CFU[d]**
1	+10	+25	+139	+100	+243	+350
2	+15	+50	+147	+200	+343	+300
3	+48	+50	+143	+ 328	+538	+614

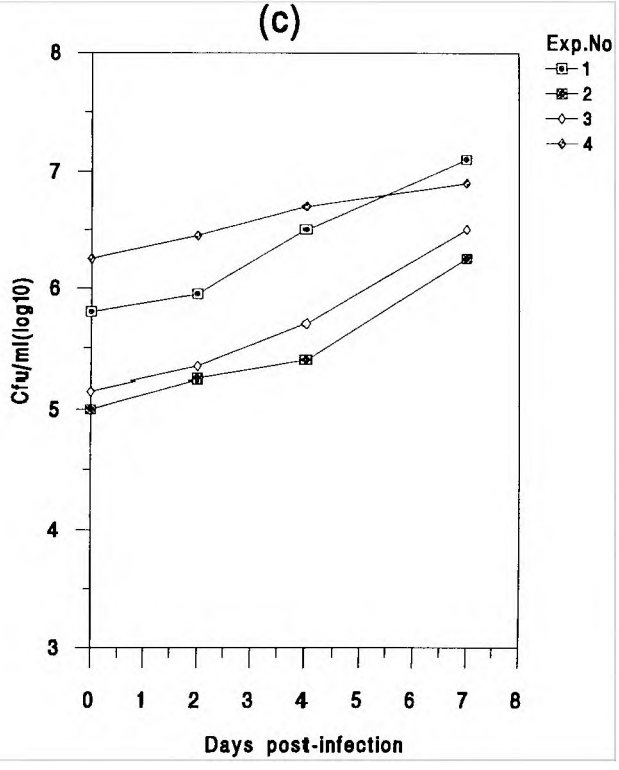
[a] Percentage change in bacterial growth is calculated by using the following equation:-
% change = [1- CFU at day (n) / CFU at zerotime (control)] x 100,
n = day post-infection. Zero-time counts (4 hours post-infection) is taken as control.
[b]Macrophages.
[c] [^{3}H] uridine incorporation expressed as counts per minute (cpm).
[d] Colony forming units (CFU) estimated by the microcolony counting technique as described in the methods section 2.2.5.
+ (Growth enhancement).

This table describes the comparison of two different methods of assessing growth/viability of BCG. 4-day old human monocyte-derived macrophages from three different donor sources were infected with 5 BCG/cell. Extracellular BCG were removed, and intracellular bacteria determined at 4 hrs post-infection as zerotime (0). BCG growth was followed over 2, 4, and 7 day post-infection. Percentage change in bacterial growth was then monitored by both methods.

Figure 2.7
Intracellular BCG growth within human monocyte-derived macrophages from different donors







Figures 2.7 (a-c)

These figures illustrate intracellular growth of BCG within 4-day old human monocyte-derived macrophages obtained from Donor 1 (a), Donor 2 (b) and Donor 3 (c). The growth/viability of BCG was determined by microcolony method (a-c) and is represented in $\log_{10}$ as an estimate of CFU/ml (x–axis). A time course of zeroime (0) i.e 4 hour post-infection and 2, 4 and 7 days post-infection was followed (y-axis). The growth assays were performed at different days, and each figure is representative of seven experiments performed on macrophages from donor 1 and 2 respectively, and four on donor 3. Each data point is a mean of triplicates +/- SD < 5% of mean (results not shown).

Table 2.6
Variation in the intracellular BCG growth within 4-day old monocyte-derived human macrophages as determined by colony forming unit (CFU) estimate

A) Day to day variation

		CFU (Log_{10}) / 5 x 10^4 Mϕ[a]			
[a]Mϕ donors	Days post-infection	Mean[b]	SD	CV[c]	% Δ[d]
1	0	5.47	0.66	12	
	2	5.69	0.66	11.6	+65
	4	6.07	0.53	8.7	+298
	7	6.57	0.53	8.8	+1150
2	0	5.82	0.27	4.6	
	2	6.02	0.32	5.3	+58
	4	6.35	0.32	5	+238
	7	6.73	0.33	4.9	+712
3	0	5.55	0.58	10.4	
	2	5.75	0.55	9.5	+58
	4	6.07	0.62	10.2	+231
	7	6.68	0.32	4.8	+1340

[a]Macrophages.
[b]Mean of 21 replicates in case of donor 1 & 2, 12 for donor 3.
[c]Coefficient of variance = [Standard deviation (SD) / mean] x 100.
[d]Percentage change in bacterial growth = [1 - CFU count at day (n) / CFU count at zerotime] x 100
n = Day post-infection.
+ (Growth enhancement).

B) Donor to donor variation

Days post-infection	Mean[f]	SD	CV[c]	% Δ[d]
0	5.61	0.18	3.2	
2	5.82	0.17	2.9	+62
4	6.16	0.16	2.6	+254
7	6.66	0.08	1.2	+1020

[f]Data obtained from 3 donors.
[b]Coefficient of variance = [Standard deviation (SD) / mean] x 100.
[d]Percentage change in bacterial growth = [1 - CFU count at day (n) / CFU count at zerotime] x 100
n = Day post-infection.
+ (Growth enhancement).

This table illustrates CFU estimate of intracellular BCG growth expressed in $\log_{10}$ values. Figures depicts day to day (A) and donor to donor (B) variation, expressed as coefficient of variance (CV), for intracellular BCG growth within human monocyte-derived macrophages. 4-day old human macrophages (5 x 10^4) were infected with 5 BCG/cell. Extracellular bacteria were removed and intracellular bacteria determined at 4 hrs post-infection as zerotime (0). Data in Table (A) is obtained from colony forming units (CFU) estimate of intracellular BCG in 7 different experiments for donors 1 & 2 each, and four experiments for donor 3. Each experiment was performed in triplicate. Data in Table (B) is obtained from CFU values in Table (A), where all three donors are compared with each other at each time point post-infection of the assay.

2.4 Discussion

Research on mycobacterial infections is being held back by lack of suitable, accurate and rapid methods of determining bacterial growth and viability. In this study a comparative assessment was made of the sensitivity of the various methods currently available. A new technique, "microcolony counting" in which BCG colonies are grown in Middlebrook 7H9 broth, was also evaluated and found to have a number of advantages over standard agar culture methods. Broth culture was shown to be as sensitive; in determining the CFU concentration of a BCG stock, as either plating out on 7H10 or Dubos Oleic agar. It also required only half the time it takes to plate out on agar. Broth culture is also a less cumbersome and very economical system and has the additional benefit of allowing a large numbers of samples to be processed in parallel on a single microtitre plate. Although assessment of mycobacterial growth by CFU determination is a much more laborious and time consuming method than radio-isotope labelling, it does provide a measure of the exact numbers of the surviving organisms. The only major disadvantage of CFU determination methods over radio-isotope incorporation, besides the time factor, is that they can be prone to aggregation errors, unless the organisms are sonicated or detergents added to the culture media. Radiometric assays however are insensitive to such clumping effects, as they are dependent on only mycobacterial metabolism.

BCG, when maintained in *in vitro* media was found to readily incorporate the following radio-isotopes: [^{3}H] uracil, [^{3}H] uridine, and [^{3}H] glycerol. The rate of incorporation into macrophage-treated bacilli however was found to be sharply reduced following cell lysis. This may be associated with the hypothesis that mycobacteria released from cells undergo a lag-phase growth [Stach *et al* , 1984]. It is known that 80% of all radio-nucleotides i.e. [^{3}H] uracil and [^{3}H] uridine, fed into mycobacterial cultures incorporate into RNA, while the remainder label the DNA fraction, while [^{3}H] glycerol labels the cell wall. The kinetics of incorporation of radio-nucleotides into bacteria in the macrophage-free system follows the curve that presumably corresponds to the kinetics of bacterial RNA synthesis in the logarithmic phase of growth [Somogyi *et al* , 1983]. The use of [^{3}H] glycerol incorporation assays in assessing *M. avium* [Blanchard *et al*, 1990], and [^{3}H] uridine in assessing *Candida albicans* viability [Bridges *et al*, 1980] have been previously reported but no one to our knowledge has previously described their use with BCG. In this study

we have demonstrated that both [^{3}H] uridine and [^{3}H] glycerol incorporate well into macrophage-free and macrophage-treated cultures of BCG.

Radio-labelling of bacteria has both advantages and disadvantages. The major disadvantage is, that it is only an accurate indicator of cell division, if it is assumed that the mycobacteria are healthy and viable. It is conceivable however that the bacilli; may become modified following passage through the macrophage, possibly taking up the label but not dividing [Stach *et al* , 1984]. The use of radio-isotope assays on cell-isolated bacteria could also suffer from two possible technical errors. Firstly, enzymes released from lysed cells could continue to inhibit bacterial growth in culture. Alternatively, the cell lysate could provide additional nutrients or a culture milieu that may promote bacterial growth. The former possibility would then lead to falsely elevated and the latter, falsely depressed growth inhibition results. Such considerations necessitate the requirement for CFU counts to be performed in parallel with the radiometric assays until the parameters of the system are fully determined.

Reports of certain mediators modulating intracellular mycobacterial growth and causing statistically significant reductions in bacterial incorporations of radiolabels compared to controls over a culture period, may not in fact indicate that protective mechanisms are operating [Rook *et al*, 1981]. Depending upon the assumed normal replication time of the macrophage-released mycobacteria, which varies from 8-24 hours, then after 3 days in culture, the percentage deficit of mycobacteria would represent a reduction of only one generation in 3 or 4. Such an effect was illustrated in a recent report in which an approximate 10-fold reduction in the number of mycobacterium was determined, following assessment by [^{3}H] uracil uptake, or by counting CFU [Kaufmann & Flesch, 1990]. However if it is assumed that *Mycobacterium bovis* divides every 8-12 hours, then this finding would also be consistent with the effect of bacteriostasis.

The use of radio-isotope incorporation as a method for assessing mycobacterial growth, as reported previously [Rook and Rainbow, 1981] has the major advantage of being very rapid and has been successfully applied in both human and murine systems [Kaufmann & Flesch, 1990, Rook *et al*, 1986, Rook & Rainbow, 1981, Flesch & Kaufmann, 1987, Blanchard *et al*, 1991]. Mycobacterial growth is then monitored indirectly by the uptake of the radio-nucleotide into mycobacterial RNA. The technique therefore

is unable to distinguish between bacterial stasis or killing, hence no conclusions about the absolute numbers of surviving mycobacteria can be made. In this study we were unable to devise a practical method of correlating the number of bacteria added to cell cultures, with the resulting cpm obtained, following washing of the adherent monolayers, as it was found to be practically impossible to wash off the extracellular bacteria in a reproducible manner. It is suggested therefore that CFU counts should always be performed in parallel, as it is possible that the BCG may be killed, or inhibited, or show sluggish metabolic activity, following their release from macrophages.

Recently assessment of mycobacterial infection and multiplication in macrophages by polymerase chain reaction (PCR) has also been described [Barrera *et al,* 1993]. But the method is semi-quantitative and its value is debatable. The use of PCR in diagnosis of clinical samples is marred by false positives and its efficacy vulnerable. Other methods like BACTEC where ^{14}C release is measured and used as an index of proliferating mycobacteria, has a role in clinical microbiology laboratories, but its experimental use is limited, due to the cost involved.

CHAPTER 3

The effect of cytokine activation on intracellular BCG growth within human macrophages and the role of neutrophils in human mycobacterial immunity

3.1 Introduction

These studies were carried out to dissect the events involved in the effector responses of human phagocytes to invading mycobacteria. Phagocytes (macrophages, monocytes, neutrophils) employ both oxygen-dependent and oxygen independent microbicidal mechanisms to kill invading micro-organisms [Hasset & Cohen, 1989]. The relative contribution of each mechanism depends upon the host immune response and the species of bacteria phagocytosed which in turn elicits the anti-microbial capacity of the phagocytes, through cytokines released by T-cells and macrophages. Neutrophils are among the first cells to arrive at the site of infection [Bloch, 1948, Montgomery & Lemon, 1933] and the macrophage migration that follows the neutrophil influx, is also thought to result from chemotactic factors released by the neutrophils, in response to mycobacteria [Antony, 1983]. The role of neutrophils in mycobacterial immunity however is yet to be fully determined. The capacity of human neutrophils to kill *M. tuberculosis* was first reported by [Brown *et al*, 1987] and was found to be effective even in the absence of serum [Brown *et al*, 1987]. Patients with Chronic Granulomatous Disease (CGD) are known to have a defect in the NADPH oxidase pathway, resulting in their phagocytes being unable to generate oxygen radicals. Experiments were then performed in this study, to see if reactive oxygen radicals were involved in killing of mycobacteria, as CGD neutrophils/macrophages would then be predicted to be less effective killers of BCG or MAI than neutrophils/macrophages isolated from normal healthy controls.

3.2 Materials & Methods

3.2.1 Isolation of human neutrophils

Neutrophils were isolated from 10 ml samples of venous blood drawn from healthy volunteers in Li-Heparin LH/10 tubes (Sarstedt). The blood was transferred into two 10ml plastic centrifuge tubes (5ml/tube) containing 1.5ml of Dextran 110 (C, P, Pharmaceuticals, Ltd, Wrexham, U.K). The blood was thoroughly mixed with the dextran by gently pipetting to avoid air bubbles and the tubes left to sediment at 37°C for 15-25 minutes. Each leucocyte-rich upper layer was then loaded on to a 3 ml Ficoll-paque gradient, and centrifuged at 400g for 25 minutes. The gradient interface containing lymphocytes and monocytes was discarded and the neutrophil-enriched cell pellet was resuspended in 1ml of ice cold distilled water to lyse the contaminating RBC's. The cell suspension was then gently mixed for 40 seconds prior to immediate transfer to a 10ml sterile centrifuge tube containing 9ml of HBSS/MOPS (Gibco, Paisley, Scotland/Sigma, St. Louis, USA). The cell suspension was then washed twice by centrifugation at 400g for 10 minutes and the cell pellet finally resuspended in HBSS/MOPS to a concentration of 10^7 cells/ml.

3.2.2 Infection of human neutrophils

A BCG stock aliquot of known concentration and viability was thawed and sonicated in a sonicating waterbath (Heathkit) for 3 minutes. The bacteria were pre-opsonised in RPMI-1640 + 10% fresh human serum for 30 minutes at 37°C/5% CO_2, then resuspended in HBSS to a concentration of 10^8/ml. Routinely 1 ml of neutrophil suspension (10^7/ml) was then added to 1 ml of BCG suspension (10^8/ml) at a bacteria to cell ratio of 10:1. The reaction mixture was then transferred into a 25ml tissue culture flask (Flow, England) and left on a rotating shaker (G 24 Environmental incubator shaker, New Brunswick Scientific Co, Edison, N.J, USA) at 20rpm for 30 minutes at 37°C/5% CO_2 to allow phagocytosis to occur. After phagocytosis the reaction mixture was centrifuged at 100g for 4 minutes and the supernatant containing non-phagocytosed BCG was removed. The neutrophil pellet was then resuspended in 5ml of HBSS and put on a 2ml FCS gradient to remove extracellular bacteria. The gradient was then centrifuged at 1000 rpm

for 5 minutes and the supernatant media and Fetal calf serum (FCS) removed. The neutrophil pellet was then resuspended in HBSS+10% human serum and aliquoted into 4 x 2 ml tubes (Flow, England) and incubated at 37°C/5% CO_2. Samples were taken at zero-time, 30 min, 60 min and 120 min by removing 200µl aliquots of the reaction mixture, to which was then added an equal amount of 2% saponin to lyse the neutrophils. The bacterial-cell lysate was then frozen at -70°C, which does not alter bacterial viability, and thawed only once before counting. The samples were then thawed and sonicated, before being assessed for CFU by the microcolony counting method, as described in section 2.2.5.

3.2.3 Intracellular BCG growth within human macrophages following cytokine activation

Four day old human monocyte-derived macrophages were obtained from Chronic Granulomatous Disease (CGD) patients and healthy controls, as described in section 2.2.2, and pre-activated with IFN-γ (1000U/ml) and TNF-α (100U/ml) for 18 hours prior to infection with BCG or MAI. The infection of adherent monolayers was carried out as given in section 2.2.4. Briefly, a bacterial suspension giving a final infection ratio of 5 bacteria/cell was added to the cell cultures. Control cell cultures were un-activated but similarly infected. Intracellular bacterial numbers were determined at 4hrs post-infection, denoted as zerotime (i.e. time 0). Four days later the monolayers were lysed with 0.2% saponin, and the numbers of BCG released, estimated by microcolony counting method, as described in section 2.2.5, while MAI CFU estimates were performed as described in section 2.2.7.

3.3 Results

3.3.1 The role of neutrophils in human mycobacterial immunity

These assays were performed to determine the role of neutrophils in mycobacterial immunity. Neutrophils were isolated from four normal healthy donors and two Chronic Granulomatous Disease (CGD) patients. The number of intracellular BCG was then determined by CFU estimation at zerotime (i.e. time 0) at 30 minutes after infection, which was taken as the control. The ability of neutrophils from the different donors to reduce the growth/survival of BCG within the first two hours after phagocytosis was then comparatively assessed. The viability counts (CFU) performed after 1 and 2 hours post phagocytosis did not however, show any significant inhibition of BCG growth in any of the donor cultures. Neutrophils from six donors were tested in this manner and similar results were obtained with them all (Fig :3.1). Neutrophils of the two CGD patient moreover, also failed to affect the growth/survival of BCG (Fig: 3.1).

3.3.2 Intracellular BCG/MAI growth within human monocyte-derived macrophages obtained from a Chronic Granulomatous Disease (CGD) patient and a healthy control.

Four-day old human monocyte-derived macrophages from a CGD patient and a healthy control were infected with either BCG or MAI, at an infection ratio of 5:1, following pre-incubation with TNF-α and IFN-γ overnight (section 2.2.4). Control cultures not pre-incubated with cytokines, but similarly infected were also included. The number of intracellular bacteria was then also determined as zerotime (i.e. time 0) at 4 hours post-infection. Intracellular growth of bacteria was then assessed at Day 4 post-infection. The results show that both BCG and MAI grow intracellularly in both cytokine-activated and non-activated macrophage cultures, as assessed by CFU estimation (Table 3.1). The BCG released from cytokine pre-activated macrophage cultures however showed an enhanced intracellular growth in both macrophage cultures from the CGD patient (+345%) and healthy control (+313%), whereas MAI growth was enhanced only in the non-activated macrophage cultures of healthy controls and in the activated macrophages of the CGD patient (Table 3.1)

Figure 3.1
Human neutrophil-mediated BCG inhibition assay

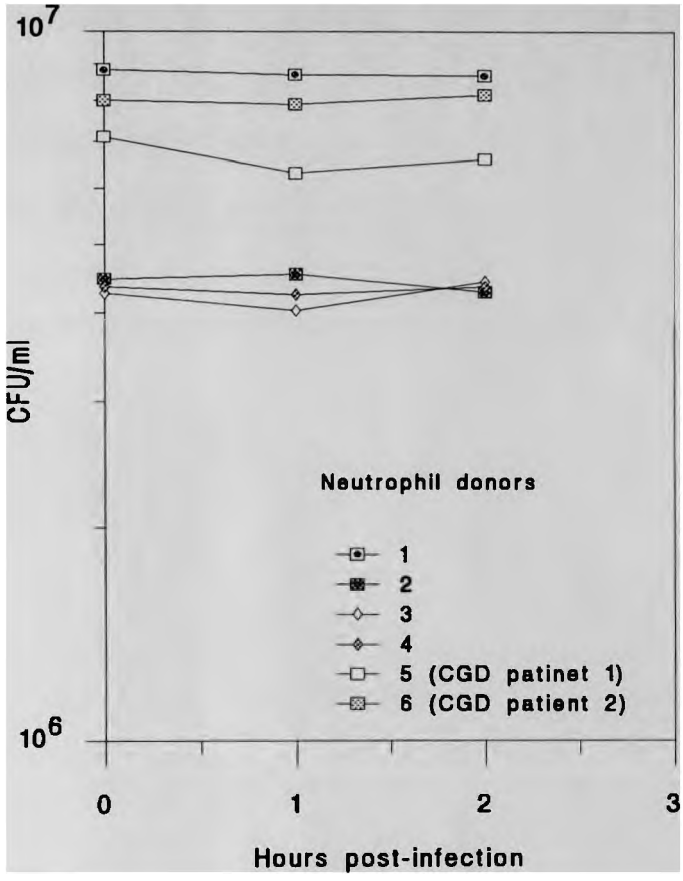


Figure 3.1

This figure illustrates the effect of incubating BCG with human neutrophils obtained from six human donor sources, including two Chronic Granulomatous Disease (CGD) patients. The neutrophils were infected with BCG at a bacteria:cell ratio of 10:1. Extracellular bacteria were removed 30 minutes post-infection and number of intracellular bacteria determined at this stage as zerotime (0). A time course of 1-2 hours post-infection was then followed. The intracellular bacteria surviving at the end of each time point of the assay was determined by estimating CFU's by the microcolony counting method. The results from four different experiments on normal healthy donors including two CGD patients are shown as CFU/ml (x–axis). Each data point is representative of mean values of 6 replicates +/- SD < 5% of mean (results not shown).

Table 3.1

Intracellular BCG/MAI growth within 4-day old human monocyte-derived macrophages from Chronic Granulomatous disease (CGD) patient and healthy control.

	Colony forming unit (CFU)/ml					
	Mϕ Control[a]			Mϕ CGD		
Cytokines[f]	Zerotime[b]	Day 4[c]	% Δ [d]	Zerotime[b]	Day 4[c]	% Δ[d]
			BCG			
-	1.6+/-0.05[e]	2.3+/-0.08	+36	5.7+/-.01	9.7+/-0.2	+70*
+	3.7+/-0.08	15.6+/-1.2	+313*	4.0+/-2.0	1.7+/-0.5	+345 *
			MAI			
-	4.3+/-0.02	11.6+/-0.5	+168*	7.7+/-0.1	1.0+/-0.02	+32
+	5.0+/-0.08	6.1+/-0.1	+21	2.5+/-.01	5.3+/-.0.4	+109*

[a]Macrophage cultures from a healthy human donor were used as control.
[b]Intracellular number of bacteria was determined 4 hours post-infection as zerotime.
[c]4 days post-infection
[d]Percentage change in bacterial growth was calculated by = [1- CFU at day 4/ CFU at zerotime] x 100 + (Growth enhancement).
[e]Data represents mean of triplicates +/-sd.
[f]Macrophage cultures were pre-activated with IFN-γ (1000U/ml) and TNF-α (100U/ml) for 18 hours prior to infection.
*Statistically significant $p < 0.5$

This table shows intracellular BCG/MAI growth within 4-day old human monocyte-derived macrophages pre-activated with cytokines for 18 hours, at the end of which they were infected with BCG at an infection ratio of 5 bacteria/cell. Intracellular bacteria were determined 4 hours post-infection as zerotime (0). At day 4 post-infection the macrophage cultures were lysed and the number of viable bacteria determined by estimation of colony forming units (CFU) by the microcolony method (section 2.2.5).

3.3.3 The effect of priming with different cytokine regimes on intracellular BCG growth within human monocyte-derived macrophages

Four-day old human monocyte-derived macrophages cultures from a healthy donor, were primed with different cytokines regimens for 18 hours prior to infecting them with BCG at an infection ratio of 5 bacteria/cell. Extracellular bacteria were then removed by repeat washing. The number of intracellular BCG was determined at zerotime (i.e. time 0) by CFU determination. Control cell cultures were included which were not activated with cytokines, but were similarly infected. The growth/viability of BCG was then determined by CFU estimation, using the microcolony counting method (section 2.2.5). The results as illustrated in table 3.2 show that pre-activation of the macrophage cultures with any of the cytokine regimes tested, did not alter the growth pattern of the intracellular bacteria. The percentage change in bacterial growth remained the same in both cytokine pre-activated and the non-activated controls.

Table 3.2

The effect of pre-activating 4-day old human macrophages with different cytokine regimes on intracellular BCG growth

		BCG (CFU/ml x 10^5)				
Cytokine pre-activation[c]	**(U/ml)**	**Zerotime[a]**	**Day 4**	**% Δ[b]**	**Day 7**	**% Δ[b]**
GMCSF	100	3.4[d]	10.2	+200	30	+723
IFN-γ	500	6.5	14.5	+123	50	+669
TNF-α	1000	2.8	7.4	+164	24	+757
IFN-γ + TNF-α	500 + 1000	3.1	8.5	+174	19	+512
Control[c]	-	5.6	12.6	+125	34	+507

[a]Intracellular BCG counts were determined 4 hours post-infection as zerotime.

[b]Percentage change in bacterial growth is calculated by using the following equation:-
= [1-CFU at day (n)/CFU at zerotime] x 100, n = day post-infection.

+ Growth enhancement.

[c]Control cultures did not receive any cytokine treatment.

[d]Data derived from 3 different experiments performed on the same macrophage donor, mean of triplicates, SD < 10% not shown.

[e]4-day old monocyte-derived human macrophages were pre-activated with different cytokine protocols for 18 hours, prior to infection with BCG.

4-day old human monocyte-derived macrophages were pre-activated with different cytokines regimens for 18 hours, at the end of which they were infected with BCG at an infection ratio of 5 bacteria/cell. Extracellular bacteria were removed by washing 4 hours post-infection. A time course post-infection was followed. At every time point the macrophage cultures were lysed and the number of viable bacteria determined by estimation of colony forming units (CFU) by microcolony method (section 2.2.5) as indicated in the table.

3.4 Discussion

3.4.1 Neutrophils do not inhibit survival/growth of intracellular mycobacteria

Patients with Chronic Granulomatous Disease (CGD) are known to have a defect in the NADPH oxidase pathway, resulting in their neutrophils and monocyte-macrophages being unable to generate oxygen radicals [Curunette & Babior 1987]. To examine the role of ROI radicals in the killing of mycobacteria in human cells, experiments were performed to assess mycobacterial growth in CGD patient-derived phagocytes. If ROI's are important mediators of inhibition of growth, or killing of intracellular bacteria, then CGD neutrophils would be predicted to be less effective killers of BCG than neutrophils from normal healthy controls. The role of neutrophils in mycobacterial immunity moreover is still controversial and has not been fully explored. Such experiments therefore were also thought to help clarify or identify an effector role for neutrophils in mycobacterial infections. It is known that in tuberculosis, neutrophils are among the first cells to arrive at the site of infection [Bloch, 1948, Montgomery & Lemon, 1933]. Moreover, the subsequent macrophage migration that follows the neutrophil influx is known to result from chemotactic factor release by neutrophils in response to mycobacteria [Antony, 1983].

The capacity of human neutrophils to kill *M. tuberculosis* was first shown by Brown *et al,* [1987] which was claimed to be effective even in the absence of serum. In his later studies however, Brown *et al,* [1987] showed that there was a good correlation between the growth index of mycobacteria in neutrophils as determined by BACTEC radiometric assay and the number of viable organisms. Using this method he reported that neutrophils from CGD patients were equally as good, as compared to normal individuals in controlling the number of viable intracellular mycobacteria, and that actual killing of mycobacteria did occur. In this study however, using the method of CFU assessment by the microcolony counting method, no evidence of intracellular inhibition or killing of mycobacteria by neutrophils was obtained.

Our results then support those previously reported by Denis (1991e), who was also unable to reproduce Anderson's findings [1991]. However in support of Anderson's findings, Sechler *et al,* (1977) and Denaver

et al, (1991) also reported that phagocytes from CGD patients showed a modest bactericidal capacity against virulent tubercle bacilli. Moreover, treatment with IFN-γ was also claimed to overcome the ROI defect in such cells [Sechler *et a,l* 1977]. The role of neutrophils then remains controversial and requires further assessment. It is of interest that apart from the question of whether neutrophils kill or do not kill mycobacteria, the lack of any effective role for ROI's in neutrophil-mediated resistance to mycobacterial infections is in fact supported by Denaver's paper who also showed that the antimycobacterial activity of normal healthy neutrophils was found to be unaffected by scavengers of free-oxygen radicals [Denaver *et al,* 1991].

Aside from neutrophils, further support for the existence of a non-oxidative antimycobacterial effector mechanism in human phagocytes, can also be drawn from clinical data on a CGD patient identified in this laboratory. The patient was documented as being immunised at birth with BCG, and subsequently developed a normal healed BCG lesion and Mantoux converted. The patient's blood monocytes and macrophages moreover were shown by *in vitro* assay, to control the intracellular growth of BCG, on par with macrophages from normal donors [R. A. Thompson & D. S. Kumararatne, unpublished observations]. There appears therefore, to be scope for further work in this area, to examine the cellular mechanisms of resistance to mycobacterial infections, using cells from such individuals.

3.4.2 Failure of cytokine-activation protocols to control intracellular mycobacterial growth within human monocyte-derived macrophages

In this study, no demonstrable inhibition of BCG or MAI growth was obtained within human monocyte-derived macrophage cultures following cellular activation with either recombinant IFN-γ, +/- LPS, or with Con-A activated lymphocyte culture supernatant (CPS), the latter used in this study as a cocktail and source of macrophage activating cytokines (Appendix 10.4). The results support previous data obtained in this laboratory which also failed to demonstrate any stimulation of macrophage-mediated killing or inhibition of growth of BCG, within infected human monocyte cell lines, Monomac-6 and THP-1 [Myint, 1992]. No inhibition was obtained in Myint's study following incubation of BCG-infected cells with a range of

cytokines, previously reported to stimulate such activity in murine macrophages, including IFN-γ, LPS, IL-1, IL-2, TNF-α, IL-6, or GM-CSF, either alone or in combination [Myint, 1992].

The results then throw into question the current dogma which states that macrophages kill intracellular mycobacteria following activation via appropriate Th1-associated cytokines such as IFN-γ, IL-2 & TNF-α [Rook, 1990a, Crowle, 1990, Kaufmann & Flesch, 1990, Lowrie & Andrew, 1988]. IFN-γ moreover has been widely reported to activate murine macrophages to inhibit the intracellular growth of mycobacteria [Reviewed in Kaufmann, 1993] and Rook *et al* (1986b), reported it to be the most potent inhibitor of *M. tuberculosis* in murine peritoneal macrophages. Conversely however, r-IFN-γ has been reported to cause only modest inhibition, or even increased growth of intracellular *M. tuberculosis* in human macrophages [Douvas *et al*, 1985, Rook *et al*, 1986c].

Our data then agrees with the current literature as concerns the role of IFN-γ in the activation of human macrophages, but still begs the question of what if anything, then activates human cells to kill mycobacteria?

There have been a number of reports in the literature of other cytokines and mediators which have been shown to activate human macrophages, including: macrophage inhibitory factor (MIF), Vitamin D, and IFN-γ. Interest in MIF re-surfaced when it was recently cloned and shown to have potent anti-mycobacterial activity in human cell systems [Orme *et al,* 1993]. Evidence has also been presented for the involvement in tuberculosis of 1, 25-dihydroxyvitamin D3, the biologically active form of vitamin D3 [Rook, 1988, Crowle *et al,* 1987]. It is has been proposed however, that it is IFN-γ that actually initially stimulates macrophages to convert 25-hydroxyvitamin-D3 to 1, 25-dihydroxyvitamin-D3 *in situ,* which in turn acts as co-stimulator of tuberculostasis. A role for Vitamin D therefore, in the lack of any direct effect with IFN-γ is questionable, unless it is proposed that some crucial metabolite(s) in the Vitamin D pathway are also required which is deficient in the *in vitro* system, and would need to be added to obtain an effect.

The possible involvement of IFN-α in the control of intracellular pathogens however may be drawn from the experiments using a *Listeria monocytogenes* infection mouse-model system, described by Havell, (1986). Furthermore, in contrast to the stimulatory role of cytokines such as IFN-γ or TNF-α in promoting anti-mycobacterial activity in human macrophages, others cytokines are now being recognised as having antagonistic roles. These include cytokines such as IL-4, IL-6, IL-10 & Transforming Growth Factor-beta (TGF-β) [Appleburg *et al,* 1992, Bermudez, 1993]. The list of factors capable of activating human phagocyte anti-microbial activity will therefore no doubt keep growing as new cytokines are continually being identified and cloned. There is therefore, still a case to be made for looking at, and attempting to identify, the cytokine signals involved in macrophage anti-mycobacterial resistance using *in vitro* model systems. Moreover, it may well turn out to be the case, that all that is required to cause human macrophages to kill mycobacteria, is to get the right balance of activators, delivered at the right stage of cell differentiation. It will be interesting to see if the current dogma stands the test of time and indeed turns out to be correct.

CHAPTER 4

The effect of TNF-α-blocking agents on BCG growth within human monocyte-derived macrophages

4.1 Introduction

Although it is generally accepted that resistance to tuberculosis is mediated by specific T-lymphocytes and expressed by activated macrophages [Hahn & Kaufmann, 1981], the cytokines involved in determining the outcome of macrophage-mycobacteria interactions in man are still unresolved. Human macrophages are known to release TNF-α following activation by IFN-γ [Havell, 1987] or after ingestion of BCG and *M. tuberculosis* [Rook *et al,* 1987, Valone *et al,* 1988, Takashima *et al,* 1990]. The latter effect is associated with the glycolipid lipoarabinomannan (LAM) cell wall component of mycobacteria [Moreno *et al,* 1989]. Moreover, it has been postulated that the toxicity of *M. tuberculosis* for macrophages may be due to the cells becoming sensitive to their own TNF, released in response to mycobacterial lipoarabinomannan (LAM), as the organism is ingested [Filley & Rook, 1991]. Experiments *in vivo* in mice with neutralising anti-TNF-α antibodies have shown a protective role for TNF-α, as neutralisation results in reduced granuloma formation and increased intracellular bacterial counts within the tissues [Kindler *et al,* 1989]. Paradoxically, injection of exogenous recombinant TNF-α *in vivo* , or treatment of BCG-infected murine macrophages *in vitro* with TNF-α appears to have little additional effect on mycobacterial growth inhibition, suggesting that mycobacteria-infected macrophages may be maximally exposed to an endogenous source of TNF-α, and are hence unresponsive to exogenous TNF-α [Kindler *et al,* 1989]. In human systems the addition of exogenous TNF-α has been shown either to have no beneficial effect [Rook *et al,* 1987] or is effective only in synergy with other cytokines [Hahn & Kaufmann, 1981], or when used against an avirulent strain of mycobacteria [Bermudez & Young, 1988]. To further elucidate the role of TNF-α in modulating human mycobacterial infections, we investigated the effect of the abrogation of endogenous TNF-α on the intracellular growth of BCG within cultured human macrophages, using four agents known to block TNF-α synthesis within macrophages. These included; Thalidomide [Sampaio *et al,* 1991], Oxpentifylline [Strieter *et al,* 1988], Dexamethasone [Remick *et al,* 1990], and an anti-TNF-α polyclonal antibody [Rahelu, 1991].

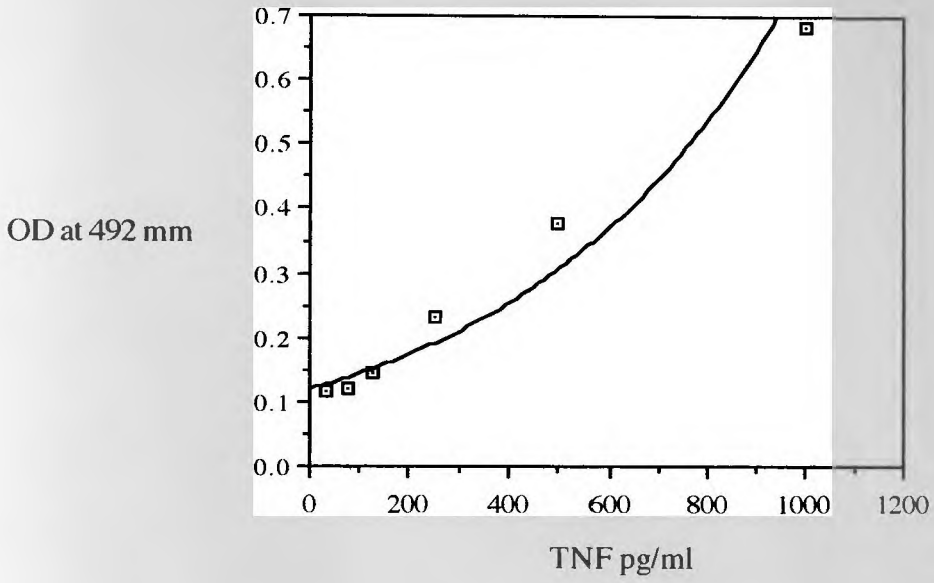
4.2 Materials & Methods

4.2.1 TNF–α ELISA assay

The concentration of TNF–α in infected macrophage culture supernatants was determined using a TNF-capture antibody ELISA assay. The assays were performed in 96-well flat bottomed ELISA plates (Nunc, Gibco, U.S.A) which had been coated overnight at 4°C with 100μl of monoclonal anti-human TNF antibody (8μg/ml, CB006, Celtech, U.K) in coating buffer (Appendix 10.2). The plates were then washed x 3 in 300μl of washing buffer (Appendix 10.2) and quenched at 1hr/37°C/5%CO_2 with a 1% BSA (Bovine serum albumin) solution in 100μl of diluting buffer (Appendix 10.2). The plates were then washed as above in washing buffer and 100μl aliquots of cell supernatants were applied to the wells in duplicate. Doubling dilutions of 100μl of Recombinant human TNF–α (Genzyme, U.K) were also added to six wells in duplicate. The plates were then incubated for 1h/37°C/5%CO_2, washed x 5 and 100μl of a second polyclonal antibody, goat anti-TNF–α (2μg/ml, British Biotechnology Limited, Birmingham) was added per well, in 2% normal mouse serum + 1% BSA in diluting buffer. The plates were then incubated for 1h/37°C/5% CO_2 and 100μl of a horseradish peroxidase (HRP)-conjugated donkey anti-sheep polyclonal antibody Ig, that binds to goat Ig (Appendix 10.2), diluted 1 : 1000 in the diluting buffer was then added and incubated for 1h/37°C/5%CO_2. 100μl of the substrate Orthophenylene diamine dichloride OPD (0.4mg/ml in substrate buffer + 0.2ml/ml of 30% H_2O_2) was then added/well and left 0.5h/37°C/ 5% CO_2. The colour reaction was stopped by adding 50μl of a 20% H_2SO_4 solution and the optical density read at 492 nm in an automated ELISA reader (Titertek Multiskan MCC, Flow laboratories, U.K). A standard curve of OD 492nm against concentration of TNF–α (pg/ml) was then plotted from which the titre of TNF–α in the unknown cell supernatants were extrapolated (Figure : 4). The sensitivity range of the TNF-α ELISA was determined to be 75pg/ml.

Figure: 4

TNF-alpha standard curve



4.2.2 Determination of the Minimal Inhibitory Concentration (MIC) of TNF–α blocking gents on BCG growth in suspension culture

To determine whether the drugs to be used to inhibit TNF–α synthesis in BCG-infected cells had growth-inhibiting properties for BCG, it was essential first to determine the critical MIC for BCG in suspension culture. The MIC, i.e. the lowest concentration of each drug which showed inhibition of BCG growth, was therefore determined for the following TNF–α-blocking agents;

1) Thalidomide
2) Oxpentifylline
3) Dexamethasone
4) Anti-TNF-α antibody

Serial dilutions of the drugs were made in 100μl of RPMI in 96-well flat-bottomed tissue culture plates (Flow, England). A stock aliquot of BCG was thawed and sonicated for 3 minutes before adjusting to two working concentration 10^4/ml and 10^6/ml in 50μl/well. Aliquots of 100μl 7H9 medium with 10% ADC supplement were also added per well to allow BCG growth. The plates were then wrapped in cling film and incubated for 14 days at 37°C/5% CO_2. A semi-quantitative visual assessment for the presence or absence of colony forming units (CFU) at each drug dilution.

4.2.3 Titration of TNF-α-blocking agents to block TNF-α release from BCG-infected human monocyte-derived macrophages

4-day old human monocyte-derived macrophages cultured in 96-well tissue culture plates were incubated with TNF-α-blocking agents with and without BCG, which was used as the stimulatory agent. BCG at bacteria:cell ratio of 5:1 and TNF-α-blocking agents were added simultaneously to the macrophage cultures. Culture supernatants were recovered after overnight (i.e. 18hrs) incubation, and TNF-α levels determined by ELISA assay. Percentage reduction of TNF-α was calculated as:100 x [1-(TNF-α experimental / TNF-α control)]; where TNF-α experimental represents TNF-α release by BCG-stimulated monocyte-derived

macrophages in the presence of TNF-α-blocking agents, and TNF-α control represents TNF-α secretion by BCG-stimulated macrophages in the absence of TNF-α-blocking agents. Monocyte-derived macrophages cultured in medium containing equivalent amounts of RPMI-1640 in the presence or absence of TNF-α-blocking agents were used as controls for TNF-α-blocking agents-treated cells. The cells were also stimulated with LPS of *Escherichia coli* serotype 026:B6 (Sigma) diluted in PBS (pH 7.4) and used at 1μ g/ml.

4.2.4 Assessment of the intracellular growth of BCG in human monocyte-derived macrophages following culture in the presence of TNF–α-blocking agents.

Macrophage cultures of PBMC-derived monocytes were prepared as described in section 2.2.2, and infected as described in Section 2.2.4. Briefly, the cultured macrophages were infected at a BCG to cell ratio of 5:1 and incubated at 37°C/5% CO_2 for 4 hours to allow phagocytosis. The TNF–α-blocking agents were added at the time of infection. The non-phagocytosed bacteria were removed by repeated washing, and the cells finally resuspended in 100μl aliquots containing the TNF–α-blocking agents. Zero-time samples were taken after 4 hours of phagocytosis, by lysing the macrophage monolayers with 20μl of 2% saponin, which had been previously shown not to have any effect on BCG viability. Aliquots of 100μl of 7H9 medium supplemented with 10% ADC were then added to each well. The growth/viability of the surviving bacteria was then assessed over a time course by labelling with [^{3}H] uridine (section 2.2.7) and by determination of colony forming unit's by the microcolony counting method (section 2.2.5). Similarly samples at day 2, 4, and 7 post-infection were taken and bacterial growth and viability similarly determined.

4.2.5 Statistical evaluation

Student's *t*-test was employed to determine the significance of growth enhancement or reduction of intracellular BCG.

4.3 Results

4.3.1 Determination of TNF–α production following BCG infection of human monocyte-derived macrophages

TNF–α production was determined following infection of 4 day cultured monocyte-derived macrophages at different bacteria to cell ratios using BCG as the infectious agent and stimuli for TNF–α production. TNF–α release was then determined by TNF–α ELISA assay (Methods 4.2.1). The results show that increasing the bacterial to cell ratio resulted in a corresponding increase in TNF–α production with BCG (Table 4.1). TNF–α production peaked (>20ng/ml), at BCG to cell ratio of 20:1. Un-activated cell cultures, and BCG grown in culture medium was not found to release any significant amount of TNF–α (Table 4.1). The tabulated results express the TNF–α concentration in ng/ml because these experiments were performed when we were in a process of standardising our TNF–α ELISA. The later experiments then show TNF results in pg/ml as the sensitivity of the assay had by then been improved.

4.3.2 Minimal Inhibitory Concentration (MIC) of the TNF–α-blocking agents as determined for BCG.

The MIC for each of the TNF–α-blocking agents, i.e. Thalidomide, Oxpentifylline, Deaxmethasone and Anti-TNF antibody were successfully determined at two standard bacterial concentrations, i.e. 10^4 & 10^6 bacteria/ml and detailed in Table 4.2. The results show that all the drugs had growth-inhibiting properties at the higher concentrations tested. Therefore the concentrations of TNF–α-blocking drugs used in all subsequent experiments were such that they blocked significant TNF-α release from BCG-infected monocyte-derived macrophages without directly inhibiting intracellular BCG growth or viability.

Table 4.1

Production of TNF-α by BCG-infected human monocyte-derived macrophages

BCG:cell ratio	TNF-α (ng/ml/5x10^4 Mϕ [a])
40:1	>20[b]
20:1	>20
10:1	18
5:1	3
2:1	1.25
Macrophage cultures alone	0
BCG cultures alone	0

[a] 4 day-old human monocyte-derived macrophages
[b] Data obtained from mean of triplicate samples from the same donor source of monocyte-derived macrophages.

4-day old human monocyte-derived macrophages were infected with different bacteria:cell infection ratios of BCG to stimulate the production of TNF-α. The levels of TNF-α (ng/ml) were determined in the culture supernatants by ELISA assay 18 hours post-infection.

Table 4.2

***Minimal Inhibitory Concentration (MIC) dilution table of TNF-α-blocking drugs for BCG**

BCG (CFU/ml)	**10^4**	**10^6**	**10^4**	**10^6**	**10^4**	**10^6**
TNF-α-blocking drugs (μg/ml)	**Dexamethasone**		**Oxpentifylline**		**Thalidomide**	
1024	-[b]	-	ND[a]	ND	ND	ND
512	-	+	ND	ND	ND	ND
256	+	+	ND	ND	ND	ND
128	+	+	-	-	ND	ND
64	+	+	-	-	ND	ND
32	ND	ND	-	+	-	+
16	ND	ND	+	+	+	+
8	ND	ND	+	+	+	+
4	ND	ND	ND	ND	+	+
2	ND	ND	ND	ND	+	+

MIC values

BCG (CFU/ml)	**10^4**	**10^6**
Dexamethasone	512 μg/ml ($1.3x10^{-6}$M)	1024 μg/ml ($2.6x10^{-6}$M)
Oxpentifylline	32 μg/ml	64 μg/ml
Thalidomide	32 μg/ml	>32 μg/ml

*Since BCG forms discernible microcolonies (CFU) after 12-14 days, when grown in 7H9 culture broth, visual assessment of the presence (+) or absence (-) of microcolonies can be made to determine the Minimum Inhibitory Concentration (MIC) of the TNF-α-blocking drugs for BCG.

[a] not determined.

[b] + (Presence), - (Absence) of bacterial growth.

4.3.3 Titration of TNF–α-blocking agents to block TNF–α release from BCG-infected cells.

Four-day old monocyte-derived macrophages were infected with BCG at a bacteria-to-cell ratio of 5:1 to stimulate TNF-α release. The system was used to titrate the effectiveness of the various agents to block TNF-α production in overnight (18hrs) cultures. The minimum concentration of each of the four agents which effectively blocked TNF-α release from BCG-infected macrophages was then determined. The results (Table 4.3) show a progressive reduction in TNF-α release by BCG-infected macrophages in the presence of increasing concentrations of the four TNF-α-blocking agents. Similar results were obtained with cells obtained from all three donors. All four agents were found to be effective at appropiate concentration in blocking exogenous TNF-α release by BCG-infected cells. The anti-TNF-α antibody was found to be the most effective in blocking TNF-α release producing 85-100% reduction in detectable TNF-α from BCG-infected cells from all the three donors. Within the MIC range of the three anti-TNF-α drugs tested (Table 4.2) dexamethasone was found to be the most effective, resulting in 84.5-100% reduction in TNF-α supernatant titres at 10^{-7}M (Table 4.3). Thalidomide was shown to be the least effective agent *in vitro*, producing only 8% (donor 2), and 50% (donors 1 & 3) reduction at 10μg/ml in TNF-α production from BCG-infected cells.

4.3.4 Effect of TNF–α-blocking agents on intracellular BCG growth within human monocyte-derived macrophages

The effect of selective TNF–α-blockade on intracellular BCG growth within monocyte-derived macrophages, obtained from three different human donors, was examined. In these experiments the bacteria to cell infection ratio used, was 5 :1. The TNF–α-blocking agents were added at the time of infection and were present throughout the assay. Monocyte-derived macrophage cultures were given 4 hours of incubation with the BCG to allow phagocytosis of the bacteria. Non-phagocytosed bacteria were removed and fresh medium containing the various TNF–α-blocking agents were re-added. The number of infecting bacteria was determined at 4hr post-infection as zerotime[time 0]. The effect of TNF–α blockade on intracellular BCG growth was then followed over a time course of 2, 4 and 7 days post-infection. The growth and viability of the intracellular BCG was then determined by two methods, i.e. [^{3}H] uridine incorporation (Fig:

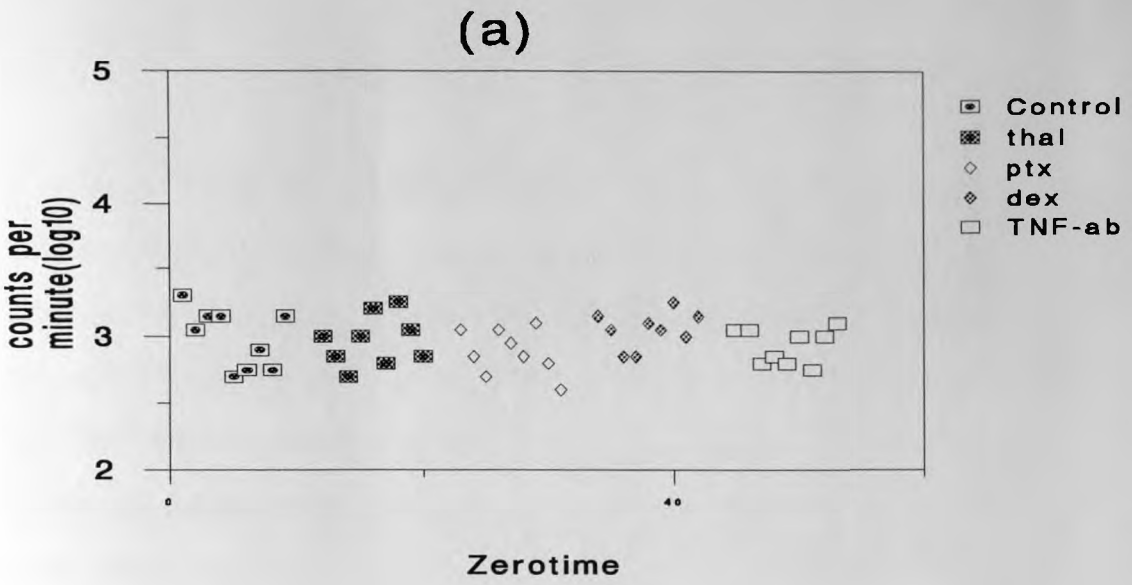
Table 4.3
The effect of TNF-α-blocking agents on TNF-α release by BCG-infected 4-day old human monocyte-derived macrophages as determined by ELISA assay

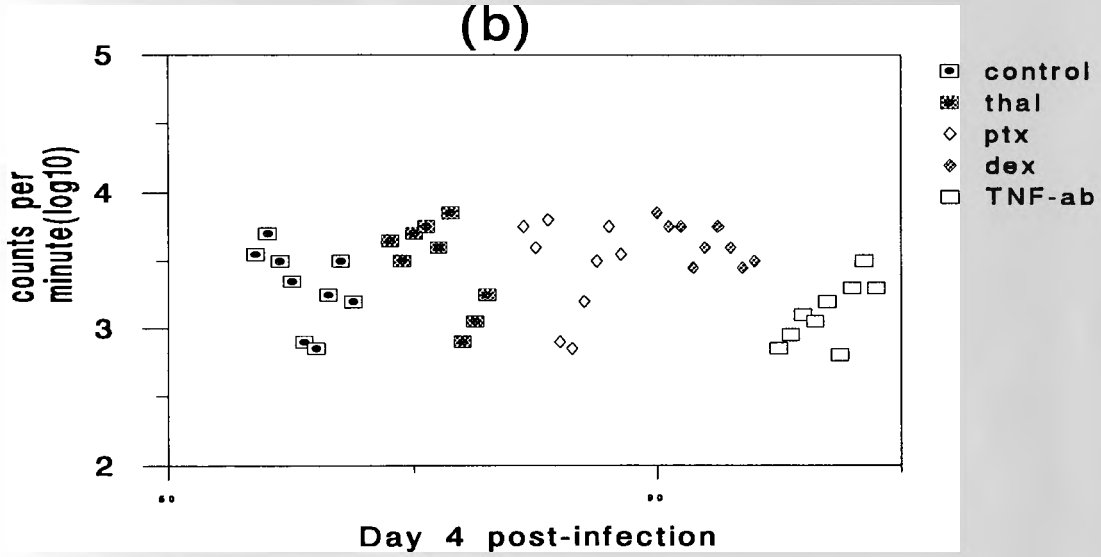
TNF-α-bloc -king agent	Dil/conc	[a]Mϕ Donor 1		Mϕ Donor 2		Mϕ Donor 3	
		[b]TNF	% Reduction	TNF	% Reduction	TNF	% Reduction
Anti-TNF-antibody							
	1:500*	75	93.84	0	100	150	85
	1:5000	418	65.17	650	8	ND	
	1:50000	650	45.84	710	0	ND	
Thalidomide							
(μg/ml)	10*	600	50	650	8	500	50
	1	750	37.5	520	26	ND	
	0.1	900	25	700	0	ND	
Oxpentifylline							
(μg/ml)	100	324	73	250	65	400	40
	10*	600	50	650	8	ND	
	1	850	29.2	600	15	ND	
Dexamethasone							
(Molar)	10^{-5}	186	84.5	0	100	0	100
	10^{-7}*	186	84.5	0	100	ND	
	10^{-9}	418	65.2	0	100	ND	
BCG -infected cells			1200	700		>1000	
Un-infected cells + LPS (5μg/ml)			394	1380		>1000	
Un-infected cells alone			0	0		0	
BCG in RPMI medium			0	0		0	

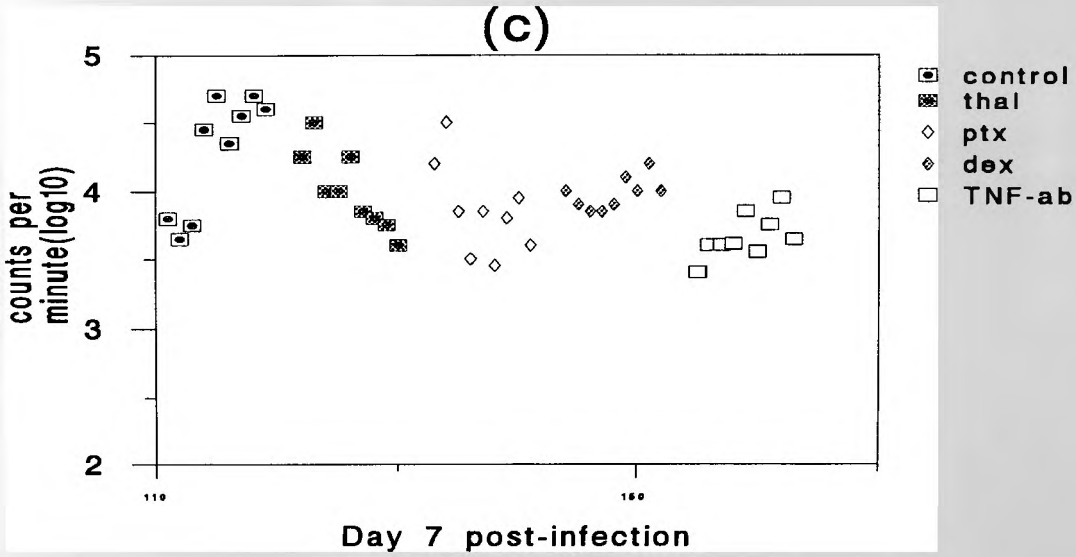
Data represent mean values of TNF-α from two different experiments. * Optimal effective concentrations used in subsequent assays. ND= not determined.
[a]Macrophage donors. [b]TNF-α is expressed in pg/ml/5x 10^4 cells. % Reduction calculated as given in methods section 4.2.3.

4.1) and microcolony counting method (Fig: 4.2), respectively. The results show that none of the four TNF–α-blocking agents had any significant enhanced the intracellular growth of BCG within human monocyte-derived macrophages (Fig: 4.1 & 4.2) as compared to control cultures in the absence of TNF–α-blockade. In fact, two TNF–α-blocking agents, dexamethasone ($p < .0007$) and anti-TNF-α antibody ($p < .0001$) actually reduced the CFU of BCG at zerotime (4 hours post-infection), and at day 2 post-infection, as determined by either method (Figures 4.1 & 4.2). This may have been the result of a reduced uptake of BCG by phagocytosis, which then affects subsequent growth of intracellular BCG over time. Anti-TNF-α antibody ($p < .0001$) also had a significant inhibitory effect on bacterial growth at day 4 post-infection. Comparable results were obtained with both methods of assessing mycobacterial growth, i.e. radiometric and CFU determination.

Figure 4.1
The effect of TNF-blocking agents on intracellular BCG growth as assessed by [3H] uridine incorporation



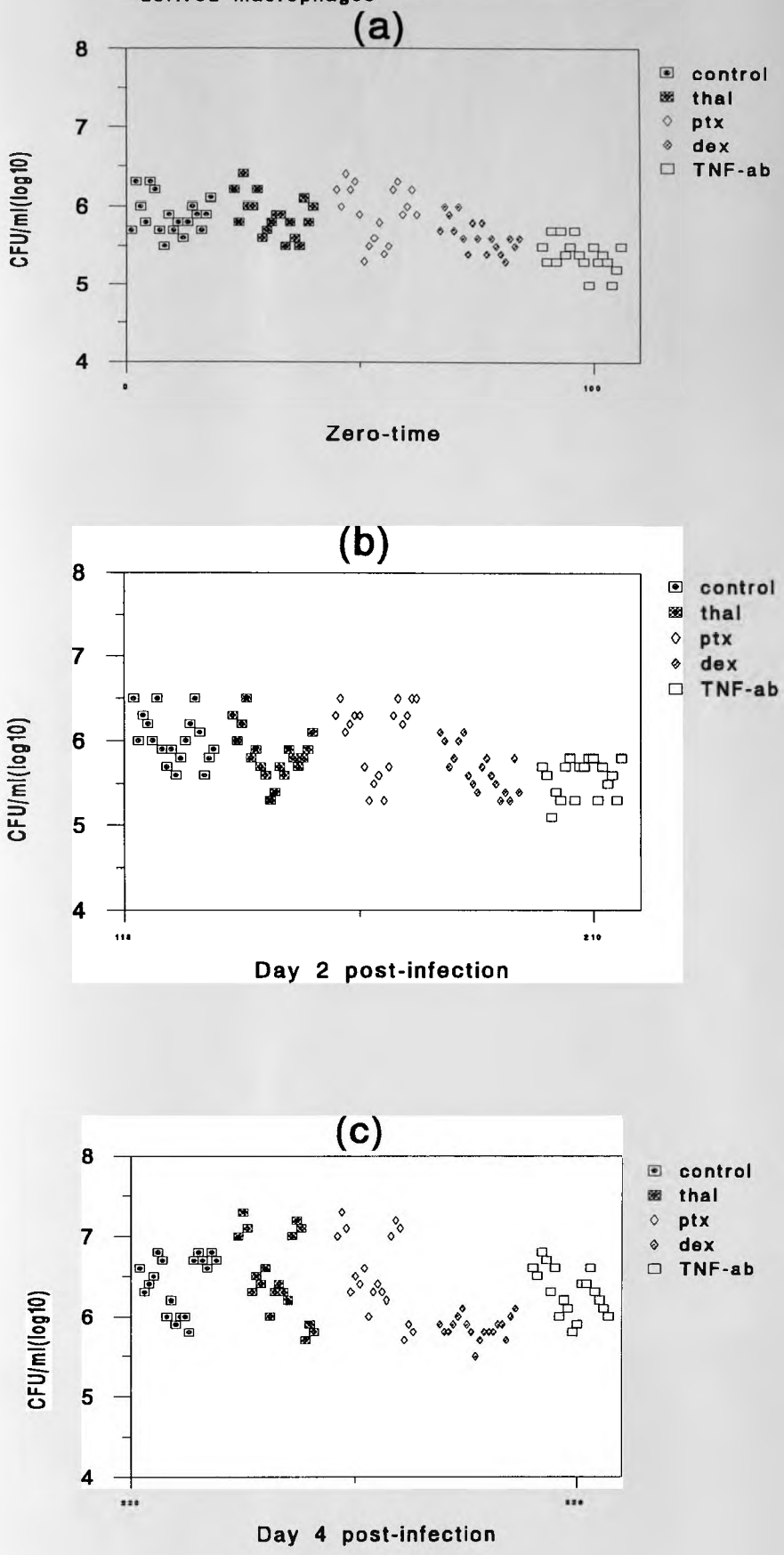


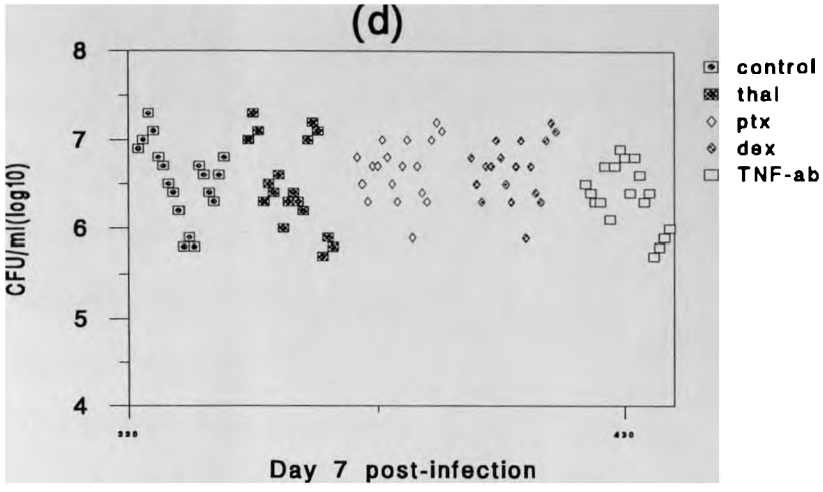


Figures 4.1 (a-c)

These figures show the effect of TNF–α-blocking agents on intracellular BCG growth within 4-day old human monocyte-derived macrophages as determined by [^{3}H] uridine incoporation over 3 days. TNF–α-blocking agents were added at the concentrations that effectively blocked TNF–α. The control cultures did not receive any TNF–α-blocking agent. The macrophage cultures were infected with BCG at a infection ratio of 5:1 and after 4 hours extracellular bacteria were removed. The intracellular number of BCG was determined as zerotime (0). A time course of 2, 4, and 7-day post-infection was then followed (y–axis). The results are expressed as $\log_{10}$ values of counts per minute along x–axis in an experiment in which three different macrophage donors were tested in triplicate. Each column represents 9 data points for each TNF–α-blocking agent tested per donor.

Figure 4.2
The effect of TNF-alpha blocking agents on intracellular BCG growth in human monocyte -derived macrophages




(d)
8
7
6
5
4
CFU/ml(log10)
Day 7 post-infection
control
thal
ptx
dex
TNF-ab

Figures 4.2 (a-d)

These figures show the effect of TNF–α-blocking agents on intracellular BCG growth within 4-day old human monocyte-derived macrophages as assessed by the microcolony method. TNF–α-blocking agents were added at concentrations that had previously been determined to effectively block TNF–α release. Control cell cultures which had not received any TNF–α-blocking agent were also included. The macrophages were infected with BCG at an infection ratio of 5 bacteria/cell and after 4 hours extracellular bacteria were removed. The intracellular number of BCG was determined by estimating CFU's at zerotime (0). A time course i.e. 2, 4, and 7-day post-infection was then followed (y–axis). The results expressed as $\log_{10}$ values of CFU/ml and depict pooled data from two experiments, in which three donors were tested in triplicate. Each column represents 18 data points for each TNF–α-blocking agent per donor.

4.4 Discussion

The role of TNF–α in anti-mycobacterial immunity has generated much interest over the past few years but its precise function is still unresolved. The purpose of this work was to analyse the role of TNF–α in controlling intracellular BCG growth within human monocyte-derived macrophages. This was achieved by blocking endogenous TNF–α release by various TNF–α-blocking agents. Since the BCG survive within human macrophages, they provide a good model to investigate anti-mycobacterial activity. The results presented here indicate that TNF–α as such has no protective or detrimental role on intracellular BCG survival within human monocyte-derived macrophages.

This study contradicts some controversial *in vivo* [Kindler *et al*, 1989] and *in vitro* [Bermudez & Young, 1988] evidence that TNF–α may play a role in determining resistance to mycobacterial infections in human cells. For example, TNF–α treated macrophages were found to kill 99% of an infecting avirulent strain of *M. avium* [Denis, 1991]. Moreover, TNF–α and IL-2 were similarly reported to have a beneficial synergistic effect in controlling an avirulent *M. avium* infection in human cells [Bermudez & Young, 1988]. In the latter study at least, the strain of *M. avium* used was derived from an AIDS patient and this had a poor replicating potential in healthy donors. As the majority of *M. avium* strains derived from non-HIV patients, however, are known to be virulent for normal human macrophages [Ellner, 1990, Crowle, 1990], such results may be attributable to the atypical organism utilised. In *M. tuberculosis* infections, however, it is thought that different strains have differing susceptibilities to the growth inhibiting effects of IFN–γ activated macrophages [Havell, 1987]. One explanation for these differences might also be a variation in the capacity of different mycobacterial strains to induce a TNF–α response in macrophages. Such an effect was found when Lipoarabinomannan (LAM) from the cell wall of mycobacterial species was found to produce different levels of TNF–α production [Chatterjee *et al*, 1993]. However, in one study; using murine macrophages, the addition of exogenous TNF–α to infected cells was not shown to have any detrimental effect on the growth of a virulent strain of *M. tuberculosis* [Rook, 1990].

In our laboratory we have shown that BCG-infected human monocyte-derived macrophages release high levels of TNF–α *in vitro*. However, in similar studies with *Mycobacterium avium intracellulare* (MAI)-infected cells, we failed to elicit significant TNF-α release, even at infection ratios ten times greater than those resulting in maximum TNF-α release from BCG-infected cells [Results not shown]. Addition of exogenous TNF–α to MAI-infected cells might then be expected to have a beneficial effect on the intracellular inhibition of bacterial growth, which would not be appreciable with a BCG infection where endogenous TNF production is stimulated. it is of interest in this context that *M. tuberculosis,* like BCG, is also known to stimulate TNF–α release from human macrophages both *in vivo* and *in vitro* [Rook *et al*, 1987, Valone *et al*, 1988, Takashima *et al*, 1990]. The charactistic pathological features of tuberculosis such as fever, weight loss and tissue damage are attributed to the release of TNF–α by infeceted cells [Rook, 1990].

The host response to mycobacterial infections may require a balance between eliciting sufficient TNF–α to promote a beneficial granuloma response, and producing too much TNF–α, resulting in tissue damage and necrosis. There have been a number of reports in which activation of macrophages has been shown to require more than one cytokine signal acting either sequentially or in synergy to promote anti-mycobacterial activity. GM-CSF and TNF–α were reported to have an additive bacteriostatic action in human macrophages infected with *M. avium* [Denis, 1991]. In *Listeria monocytogenes* infections, effective activation of macrophages required the sequential treatment of cells with both IL-2 and TNF–α [Zerlauth *et al*, 1991]. TNF–α has been reported to synergise with IFN–γ *in vitro* in the activation of both anti-parasitic and anti-tumour capacity of murine monocytes [Esparza *et al*, 1987]. It is becoming apparent however that cytokines may also promote growth of intracellular organisms [Denis & Greg, 1990, 1991]. IL-6, IL-3 and CSF may promote the intracellular growth of certain strains of *M. avium* [Denis, 1991]. GM-CSF and IL-10 have also been reported to promote, rather than restrict, the growth of some intracellular parasites [Greil *et al*, 1988, Howard & O'garra, 1992]. Thus it is possible that enhanced macrophage anti-mycobacterial activity may require other cytokines in addition to TNF–α which is not fulfilled in our *in vitro* experimental system.

Analogies may be drawn from the cytokine modulation of Nitric Oxide (NO) release within activated macrophages. In mouse cells, NO production has been clearly demonstrated to be regulated by a two-signal process, involving initial activation of cells by IFN–γ, with a requirement for either LPS or TNF–α as a second signal [Liew & Cox, 1991]. However, evidence that these cytokines act similarly in human cells is currently lacking. The cytokine mediator(s) involved in the modulation of anti-mycobacterial infections in human cells may well then be more complex than is currently suggested by extrapolation from rodent studies. Combinations of cytokines, some of which may be as yet uncharacterised, may be involved.

CHAPTER 5

Failure of the nitric oxide (NO) pathway to control the intracellular growth of five strains of *M. smegmatis* with different catalase activity within murine and human macrophages, and of NO to affect the growth of BCG in human cells.

5.1 Introduction

Nitric oxide [NO] is considered to be an important mediator involved in the killing or growth inhibition of various mycobacterial species within activated murine macrophages [Denis, 1991b, Chan *et al*, 1992, Adams *et al*, 1991, Flesch & Kaufmann, 1987], although its role in human cells is still controversial [Granger *et al*, 1988, Green *et al*, 1990, Liew *et a,l* 1990]. Moreover murine macrophages, activated with recombinant interferon-gamma [IFN-γ], tumour necrosis factor alpha [TNF-α], or endotoxin, have also been shown to kill a diverse range of other intracellular pathogens, including the parasite, *Leishmania major*, by non-oxidative L-arginine-dependent generation of reactive nitrogen intermediates. The leishmanicidal effect of murine macrophages has also been directly correlated with the release of NO [Liew *et al*, 199O, Bogdan *et al*, 1990].

The enzyme catalase has recently been shown to inhibit both the induction of nitric oxide [NO], and the NO synthase [NOS] enzyme, when added exogenously, to activated murine macrophages, leading to the inhibition of killing of *L. major* [Li *et al*, 1992]. These results could not be explained simply by the reduction of H_2O_2 by catalase, since its removal by an alternate mechanism, the addition of glutathione peroxidase had no effect on the NO synthesis or on the leishmanicidal function of activated macrophages. Furthermore, catalase did not affect the induction of NO synthase in IFN-γ activated macrophages [Li *et al*, 1992]. The inhibitory effects of catalase on NO synthesis and leishmanicidal activity, however were shown to be reversed by the addition of tetrahydrobiopterin, a co-factor of NO-synthase, in a dose-dependent manner. The effect of catalase was then proposed to be the result of oxidation of tetrahydrobiopterin [Li *et al*, 1992]. However catalase was reported not to affect NO synthesis in murine endothelial cells or neutrophils. This could be explained as induction of enzyme production of NO, the enzyme more readily pinocytosed into macrophages and thus affected the NO system more directly within the cytoplasm of such cells [Li *et al*, 1992].

Strains of *M. smegmatis*, either naturally deficient in the gene regulating catalase & peroxidase production or into which the Kat-G gene of *Mycobacterium tuberculosis* has been transfected, are available for

experimentation [Heym *et al,* 1993]. The isoniazid susceptibility of *Mycobacterium tuberculosis* is mediated by the product of the katG gene which encodes the heme-containing enzyme catalase-peroxidase [Heym *et al,* 1993]. Moreover, interest in catalase expression by mycobacteria has recently been engaged following the observation that catalase activity is reduced in INH-resistant strains of *M. tuberculosis* [Zhang *et al,* 1992]. Decreases in catalase activity seen in INH-resistant strains have also been related to reduced virulence in guinea pigs [Zhang *et al,* 1992]. Furthermore, differential susceptibility among different strains of mycobacteria to RNI has been reported [Flesch & Kaufmann, 1987, Doi *et al,* 1993] and although some mycobacteria are thought to have the capacity to reduce NO_2^- and NO_3^- [Wayne & Kubica, 1986], this may in fact be attributable to differences in catalase expression.

Infection of such strains into murine and human phagocyte cultures was then considered as a useful model system to investigate further both the effect of catalase on NO synthesis within cells, and to test whether catalase-mediated inhibition of NO synthesis correlated with reduced intracellular survival of mycobacteria within murine and human phagocytes. Five strains of *M. smegmatis* i.e. mc^2155 (wild type parental), BH1 (Spontaneous, INH-resistant mutant of *M. smegmatis* mc^2155 which is deficient for catalase-peroxidase production), and BH1/pBH4 (Original shuttle plasmid), BH1/T35 (katG+), & BH1/T586 (partially overlaps katG but does not carry the catalase-peroxidase gene from *M. tuberculosis*), [Heym *et al*, 1993] were used to infect both the murine macrophage cell line J-774 and human peripheral blood mononuclear cell-derived macrophages. The levels of NO_2^- in cell culture supernatants were then determined and correlated to bacterial survival and intracellular growth of the different strains. Possible catalase-mediated resistance to NO killing was also evaluated by assessing strain survival within cell-free cultures, containing titrations of sodium nitrite ($NaNO_2$) and sodium nitroprusside (SNP), as *in vitro* generators of NO.

The antimycobacterial effects of nitric oxide were also examined utilising BCG-infected macrophages as an experimental model system. BCG growth was then assessed in infected human cells following incubation with the L-arginine analogue, LNMMA (L-N$_G$-mono-methyl-arginine) which is known to block NO-synthase in murine cell systems. The inhibitory effects of sodium nitrite on BCG growth was also assessed in cell-free cultures.

5.2 Materials & Methods

5.2.1 Preparation of *M. smegmatis* cultures

Five strains of *M. smegmatis* obtained from Dr. S. Cole (Pasteur Institute, Paris) were initially cultured on Lowenstein-Jensen medium and then stock cultures of each prepared in Middlebrook 7H9 broth supplemented with 10% ADC supplement + 0.5% Glycerol for 48hrs. Stock bacterial suspensions were washed (x3) in RPMI-1640 by centrifugation at 2000 rpm/15 minutes and stored in 1ml aliquots at -70°C until required. An aliquot of each stock was thawed, sonicated in a sonicating water bath (Heathkit) for 3 minutes and plated on 7H10 agar + 10% ADC for 48hrs at 37°C to enumerate the CFU concentration of each strain. Note, Kanamycin 25 μg/ml (Sigma) was used in all media used to culture transfected strains to maintain the antibiotic selection for the KatG gene.

5.2.2 Intracellular *M. smegmatis* growth assays

Murine cell studies

The mouse monocyte cell line J-774 was routinely maintained in antibiotic-free RPMI-1640 medium + 10% FCS in 25cm^2 tissue culture flasks (Appleton woods, Birmingham, U.K) at 37°C/5% CO_2. The cells, when required were then resuspended by repeated pipetting and the viability of the cells determined by trypan blue exclusion. Aliquots of 10^5 cells (200μl/well) in RPMI-1640 + 5% FCS were then plated out either in 96-well flat-bottomed tissue culture plates, or at 2.5 x 10^5 cells (2ml/well) in 24-well plates and incubated overnight at 37°C/5% CO_2 to allow adherence. Non–adherent cells were removed by gentle repeat washing and fresh warm medium added. The number of adherent cells was determined as previously described (section 2.2.3). The cells were then primed with 500U/ml of murine recombinant IFN-γ (Boehringer, Germany) and 10 ng/ml of LPS (Sigma) for 4hrs. Non-activated control cultures were also included. The viability of cells was then determined by trypan blue exclusion, post-treatment with either IFN-γ and/or LPS.

Bacterial cultures were thawed and sonicated for 3 minutes, adjusted to the required concentrations, and opsonised with 5% fetal calf serum (FCS) for 0.5hr/37°C/5% CO_2. The adherent monolayers of J-774 cells were then infected at a bacteria:cell infection ratio of 5:1. The plates were again incubated at 37°C/5% CO_2, and after 24-48hrs, culture supernatants harvested for NO_2^- determination and frozen at -70°C until chemiluminiscence assessment was performed.

In a separate series of experiments bacterial viability and growth were determined at different time points post-infection. The cells were then washed to remove extracellular bacteria after a 0.5hr infection period, then, lysed with 0.2% saponin, and bacterial viability counts then determined by radiolabelling with 2μCi/well of [^{3}H] uridine for 4hrs (section 2.2.7). Colony forming unit's (CFU) were also determined by plating out cell lysates on nutrient agar for 48hrs (Methods 5.2.1).

Human cell studies

Human cell cytotoxicity assays were performed as previously described (section 2.2.4). Briefly, PBMC from human volunteers were obtained by Ficoll-paque separation (section 2.2.2). Non–adherent cells were removed after 4hrs and the adherent cells were cultured in RPMI-1640 + 10% autologous serum for 4 days at 37°C/5% CO_2. Four-day old human monocyte-derived macrophage cultures were then infected with pre-opsonised bacteria, at a bacteria to cell infection ratios of 5:1. After 0.5hr of incubation, to allow phagocytosis to occur, extracellular bacteria were removed by repeat washing and fresh medium added. A time course of 1, 2 and 3 days post-infection was then followed. At each time point the cells were lysed by 0.2% saponin, and the released bacteria enumerated by both [^{3}H] uridine labelling (section 2.2.7) and CFU estimation, as described in Methods 5.2.1 .

5.2..3 Effect of sodium nitrite (NO_2^-) and sodium nitroprusside (SNP) on different strains of *M. smegmatis*

Bacterial suspensions of each strain of *Mycobacterium smegmatis* (5 x 10^5 bacteria/ml) were incubated at 37°C/5%CO_2 with graded dilutions of sodium nitrite (NO_2^-, Sigma) or sodium nitroprusside (SNP, Sigma) pH 7.3, in 7H9 medium for 24 hours. The cells were then washed twice by centrifugation at

1000g for 15 minutes, and resuspended in fresh RPMI-1640 medium. Control cultures not treated with NO_2^- or SNP were also included. The growth/survival of the bacteria was then assessed by [^{3}H] uridine incorporation for 4hr (section 2.2.7) and by CFU determination (Methods 5.2.1) following incubation of serial dilutions of bacteria suspensions after each treatment, on nutrient agar plates for 48hrs. Kanamycin was added to cell culture media used for the transfected strains to maintain the antibiotic selection for the KatG gene.

5.2.4 The effect of LNMMA (NO-synthase blocker) on intracellular BCG growth within human monocyte-derived macrophages

PBMC's were obtained from normal Caucasian, Afro-Carribean, and Asian volunteers and from a tuberculosis and a Chronic Granulomatous Disease (CGD) patient as described in section 2.2.2. Briefly, 4-day old human monocyte-derived macrophages were cultured in 96-well flat-bottomed plates. A Conconavalin-A stimulated peripheral blood mononuclear cell supernatant (CPS) was then added, as a cytokine source to the macrophage cultures to induce NO release. L-N^G-mono-methyl-arginine (LNMMA) (Wellcome Labs, Beckenham, Kent, U.K.) was then incubated with the cells for 4hrs prior to infection of the monolayers with BCG (Copenhagen, Denmark) at an bacteria to cell ratio of 5:1. Cell supernatants were removed at various time points post-infection i.e. at 4, 18, 48, and 72 hours, and assayed for NO by chemiluminescence assay. The cultures were then lysed at different time points with 0.2% saponin and the growth/viability of BCG determined by both radio-isotope uptake (section 2.2.7) and CFU assessment as described in section 2.2.5.

5.2.4 Chemiluminiscence assay

NO_2^- levels in the culture supernatants were kindly performed in the laboratories of Professor. F.Y. Liew, Department of Immunology, University of Glasgow, Scotland.

5.3 Results

5.3.1 NO_2- release from activated J 774 cells

In a series of initial experiments, nitric oxide production by murine J774 cells was evaluated following activation by either IFN-γ and/or LPS, and/or infection with *M. smegmatis* (Table 5.1). High levels of NO_2^- (32nM/ml) were detected in culture supernatants following cell activation with IFN-γ & LPS for 24hrs (Table 5.1). Cells activated with IFN-γ alone however released relatively low levels of NO_2- (5nM/ml), similar to non-activated cells (< 3nM/ml). Increasing the time of incubation from 18 to 48hrs however resulted in a marginal rise in NO_2^- production by IFN-γ & LPS activated cells from 32 to 45nM/ml (Table 5.1).

Infection of non-activated J774 cells by any of the five *M. smegmatis* strains examined, did not result in the release of detectable amounts of NO_2^- (< 3nM/ml) over 24hrs post-infection (pi) (Table 5.1). However infection of J774 cells for 24 hrs, following pre-activation with IFN-γ (4 hrs), resulted in greater production of NO_2^- at 24hr, than that observed following activation with IFN-γ & LPS in combination i.e 40-57 nM/ml. Pre-activation of J774 cells with IFN-γ and LPS for 4hrs, followed by infection for 24hrs with any of the five strains of *M. smegmatis*, resulted in high amounts of NO_2^- (45-60nM/ml) being detectable in culture supernatants at 24hrs pi. The amounts produced however did not vary markedly between strains (Table 5.1). It is worth noting however that no significant amount of NO_2 (< 4nM/ml) was detected in *M. smegmatis*-infected cell cultures of human macrophages following any of the different regimens of inducing NO, over the time course examined (Appendix 10.3).

Table 5.1

NO_2^- production by activated J774 murine cells

J774 cells activated with			*NO_2(nM/ml/10^5 cells)	
IFN-γ (500U/ml)	LPS (10ng/ml)	*M. smegmatis* (5 bacteria/cell)	24 hrs	48 hrs
-	-	+[1]	< 3	< 3
-	-	+[2]	< 3	< 3
-	-	+[3]	< 3	< 3
-	-	+[4]	< 3	< 3
-	-	+[5]	< 3	< 3
-	-	-	< 3[a]	< 3[a]
+	-	-	5	6
-	+	+	< 3	< 3
+	+	-	32	48
+	+	+[1]	54	50
+	+	+[2]	60	55
+	+	+[3]	55	60
+	+	+[4]	45	52
+	+	+[5]	52	50
+	-	+[1]	50	53
+	-	+[2]	45	48
+	-	+[3]	57	60
+	-	+[4]	56	56
+	-	+[5]	40	45

[1]MC_2155
[2]BH1
[3]BH1/pBH4
[4]BH1/T35
[5]BH1/T586

* Data from 10 experiments performed at different days, shown as mean of triplicate values (sd < 10% of mean not shown).
+ (Presence) , -- (Absence) of activating agent.
[a](cells alone)

This table shows the nitrate levels (nM/ml/10^5 J774 cells) in the supernatant harvested from the cell cultures pre-activated with different cytokine regimens, and incubated with or without 5 different strains of *M. smegmatis* infection. NO_2^- was determined at 24 and 48 hrs post stimulation by chemiluminiscence assay.

5.3.2 Failure of induction protocols to produce NO_2^- from activated human monocyte-derived macrophages

Activation of 4-day old human PBMC-derived macrophages with recominant human IFN-γ (500U/ml) either alone, or in combination with LPS (10ng/ml) failed to elicit any significant prodution i.e. <4nM/ml of NO_2^-, as detected by the chemiluminescence assay. Moreover, no significant amount of NO_2^- i.e. < 4 nM/ml was detected in cell supernatants of human macrophages following infection with any of the 5 strains of *M. smegmatis* tested, whether the cells were pre-activated with IFN-γ or not (Appendix 10.3).

In a further study, a series of experiments was performed to investigate the inductionof NO_2^- in human PBMC-derived macrophages, with age of culture. These experiments were primarily performed to reproduce the studies of Denis [1992], who reportedy obtained demonstrable NO release from 14-day old monocyte-derived human macrophages, following infection with *M. avium*. Five sets of human monocyte-derived macrophages were then set up at different stages of maturation, i.e. 1, 4, 7, 10 and 14-days post culture, with cells from the same donor. These were then activated to stimulate NO_2^- production by either pre-activation with IFN-γ (1000U/ml), LPS (10ng/ml) or a combination of both, or with a Con-A stimulated PBMC supernatant (CPS), which was used as a crude cocktail of cytokines and/or infected with *M. smegmatis* or BCG at an infection ratio of 5 bacteria/cell. Controls which were un-infected but pre-activated with the various cytokine regimens were also included. The supernatants from the cell cultures were then collected at different time points post-infection, and nitrate levels determined by chemiluminiscence. The results show that none of the treatment regimens produced detectable levels of nitrite, i.e. < 4nM/ml following activation at any time point following infection as tabulated in (Appendix 10.4).

5.3.3 Lack of correlation between nitric oxide production by IFN-γ–activated J774 cells and their ability to inhibit the intracellular growth of different strains of *M. smegmatis*

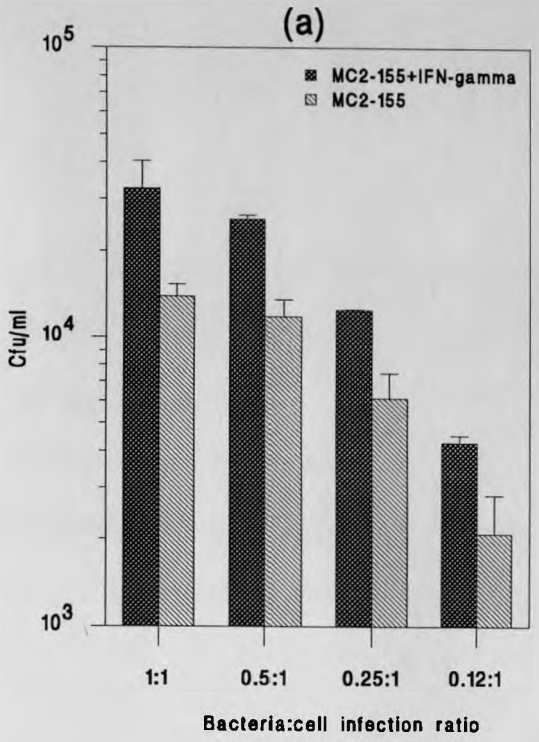
The mycobactericidal potential of nitric oxide, generated by IFN-γ-activated J774 cells was evaluated following infection against 5 different strains of *M. smegmatis* with different catalase producing abilities in parallel with measurement of NO production. The J774 cells were activated by pre-incubation with IFN-γ

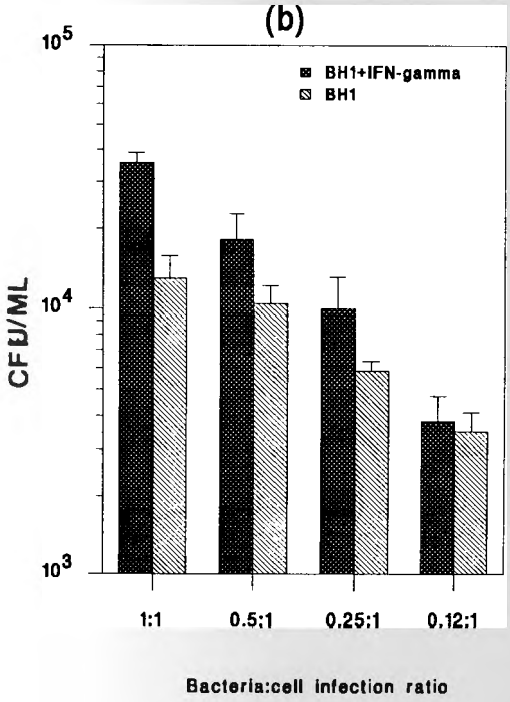
(500U/ml final concentration) plus LPS (10ng/ml) for 4hr prior to infecting them with graded doses of bacteria, which was performed by incubating the cells with the bacteria for half an hour to allow phagocytosis to occur.

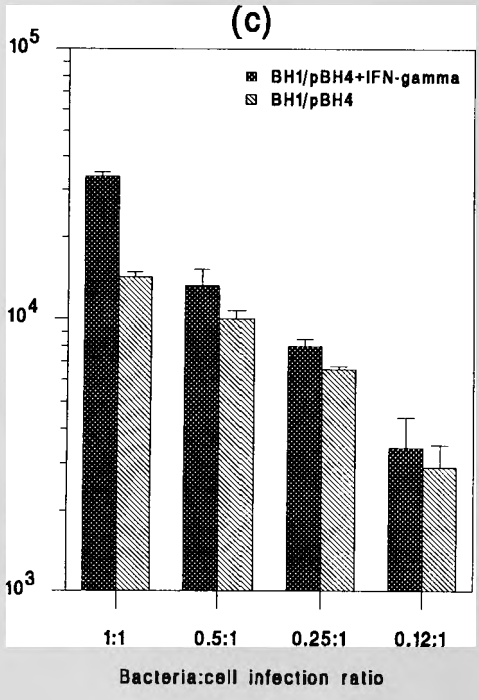
In an initial series of experiments, extracellular bacteria were not removed and the concentration of NO in the culture supernatants was assessed after 24-48h post-infection. Bacterial viability and concentration were then also determined at 18h post-infection by CFU estimation. The results depicted in figure 5.1 show that for the 3 strains of *M. smegmatis* examined i.e. MC^2-155, BH1 and BH1/pBH4, there was no inhibition of growth in any of the strains at 18hr post-infection as depicted by CFU estimation at any of the infection ratios tested (Fig 5.1). Indeed, there was an increase in the number of surviving bacteria in cultures containing IFN-γ–activated cells (Figures 5.1a-c), even though high concentrations of NO_2- were detected in the culture supernatants (Table 5.1).

Since in the previous experiments the extracellular bacteria were not removed, the possibility that extracellular bacterial growth was also being measured could not be excluded, as *M. smegmatis* unlike BCG grows in all media [Figure 5.2]. The following experiments were then performed, where extracellular bacteria were removed prior to estimation of bacterial growth with time. Two further transfected strains of *M. smegmatis,* i.e. BH1/T35 and BH1/T586 were also utilised in these experiments. IFN-γ activated J774 cells were then infected with *M. smegmatis* at an infection ratio of 5:1. The infecting number of bacteria were then determined as time [0] i.e. 4 hour post-infection and at day 3 post-infection. The remaining infected cell cultures were lysed with 0.2% saponin and CFU determined (Figures: 5.3a-e). By this method, intracellular growth was observed for all 5 strains of *M. smegmatis* within activated and non-activated J774 cells. The results suggest that IFN-γ activated J774 cells were no better than non-activated cells in controlling the growth of the 5 strains of *M. smegmatis* tested. The bacteria moreover were unaffected by the high concentrations of NO generated by these activated cells (Table 5.1).

Figure 5.1
Intracellular growth of different strains of M. smegmatis in activated/non-activated J774 murine cells







Figures 5.1 (a-c)

These figures illustrate the intracellular growth of three strains of *M. smegmatis* at various bacteria:cell infection ratios. Murine J774 cells were activated with 500U/ml of IFN-γ and 10ng/ml of LPS for 4hrs prior to infection with the strains of *M. smegmatis* at bactera:cell infection ratios of (1:1, 0.5:1, 0.25:1 and 0.12:1). Un–activated control cell cultures were similarly infected. Extracellular bacteria were not removed. The growth/viability of surviving bacteria was determined 3 days post-infection by estimating colony forming units (CFU's) by plating on 7H10 agar. Each column represents mean of triplicates +/- SD (vertical bars).

Figure 5.2
M. smegmatis (MC2-155) growth in different culture media

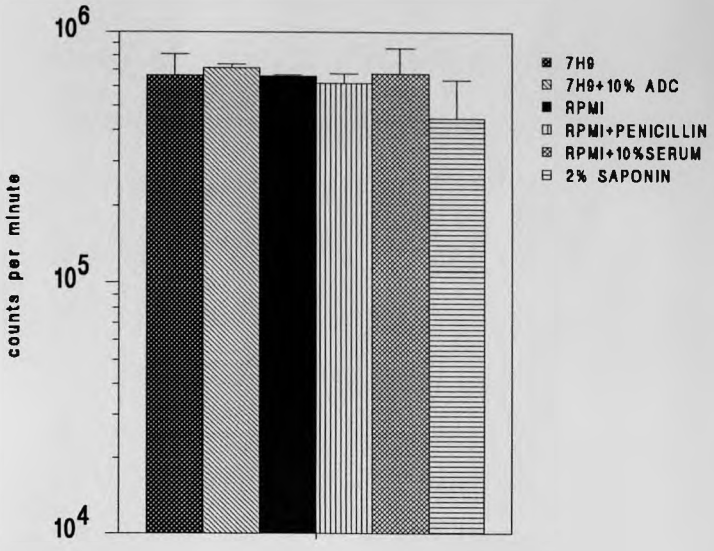
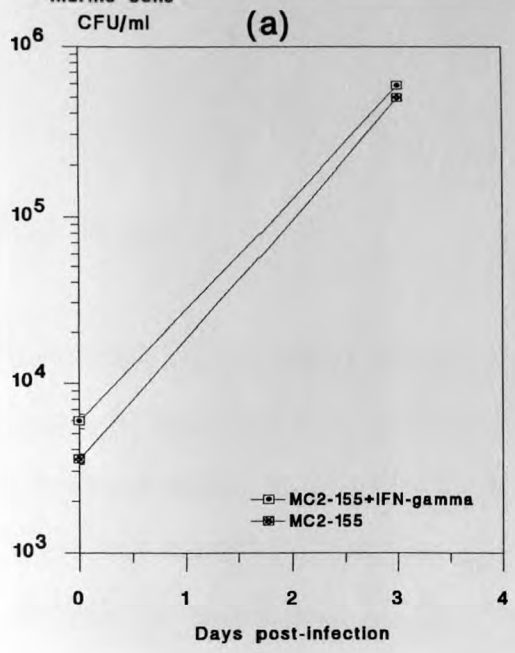
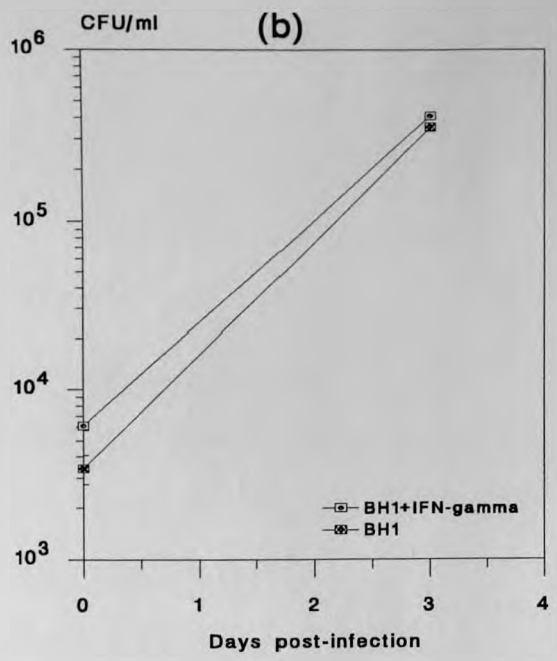


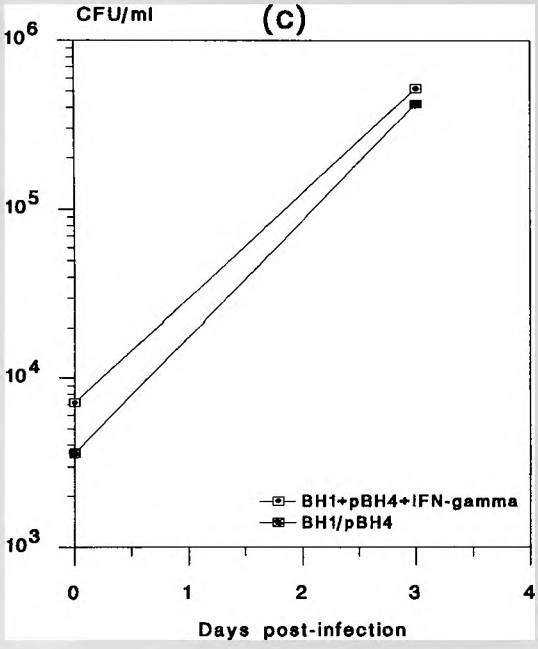
Figure 5.2

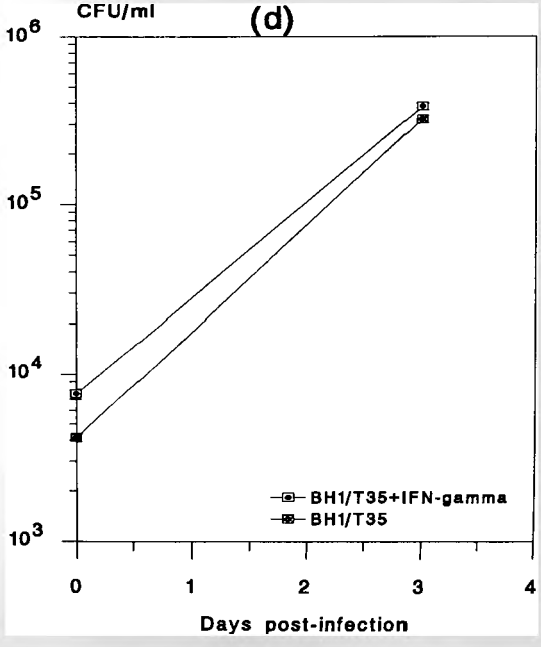
This figure depicts the growth of *M. smegmatis* (MC2-155) in different culture media as determined by [^{3}H] uridine incorporation. Aliquots of 10^5 bacteria/well were added to different culture media containing 2μCi of [^{3}H] uridine. The bacterial cultures were harvested 4 hours post-incorporation. Each column represents mean of six replicates +/- SD (vertical bars).

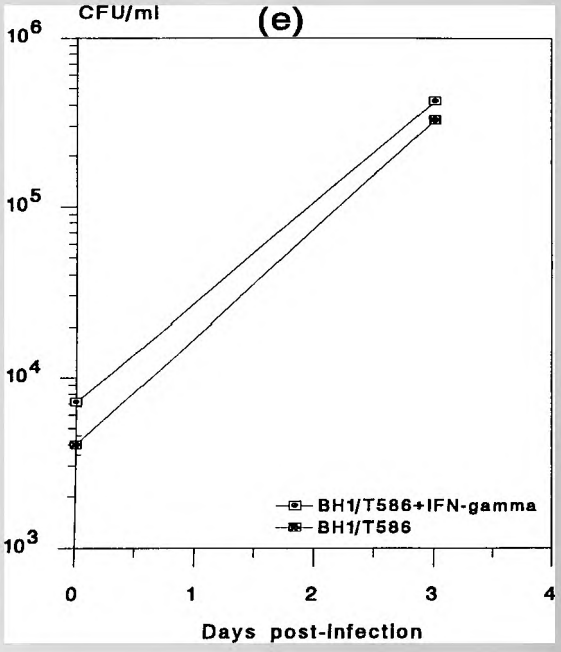
Figure 5.3
Intracellular growth of different strains of M. smegmatis within activated/non-activated J774 murine cells











Figures 5.3 (a-c)

This figure illustrates the intracellular growth of five different strains of *M. smegmatis* within activated/non-activated J774 cells, infected with 5 bacteria/cell. Extracellular bacteria were removed 4hrs post-infection. The number of intracellular *M. smegmatis* CFU was determined as zerotime (0). The infected cell cultures were then harvested 3 days post-infection, and the viability of bacteria determined by plating on 7H10 agar for 48 hours. The result in the figures represent data from six experiments performed at different days, each data point represents mean of triplicates +/- SD < 5% of mean (results not shown).

Human cell studies

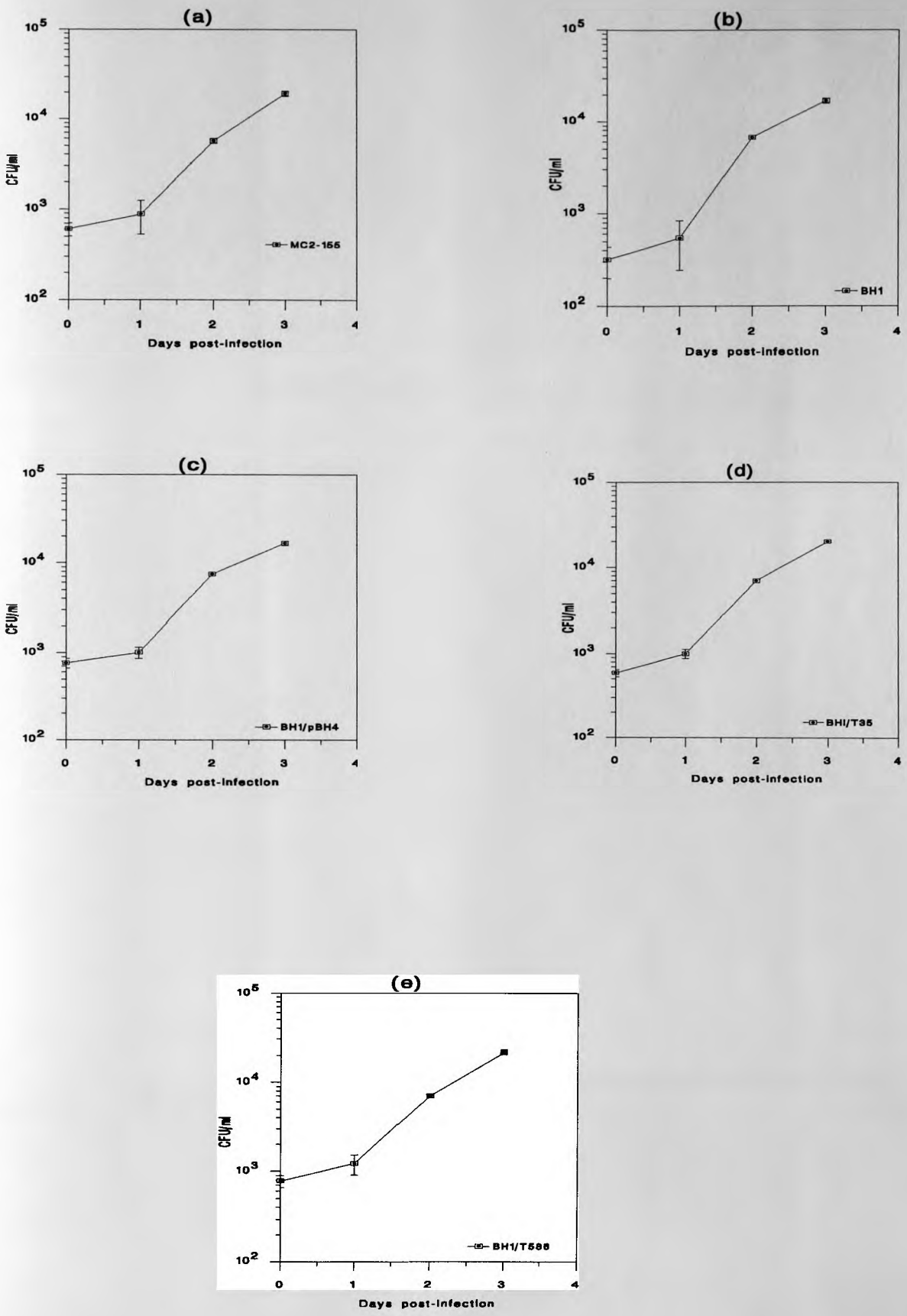
5.3.4 Intracellular growth of *M. smegmatis* strains within human monocyte-derived macrophages

Four-day old human monocyte-derived macrophage monolayers were prepared from different donors and infected with various strains of *M. smegmatis* at a bacteria to cell infection ratio of 5:1 as described (Methods 5.2.2). Extracellular bacteria were removed and the intracellular infecting number of *M. smegmatis* CFU determined 4hrs post-infection as time zero [0]. A time course of 1, 2, and 3 days post-infection was then followed to look for differences in intracellular growth rates among the different strains of *M. smegmatis* within these cells. All the strains of *M. smegmatis* i.e MC^2-155, BH1, BH1/pBH4, BH1/T35 and BH1/T586 were observed to grow well within the adherent human macrophage cultures. An approximate 2-log intracellular growth of bacteria was observed in each strain over 3 days post-infection, as determined by CFU estimation (Figures:5.4a-e). In this series of experiments the viability of the infected cells was also determined by trypan blue dye exclusion, and the results showed that the cells remained adherent and viable throughout the course of the assay.

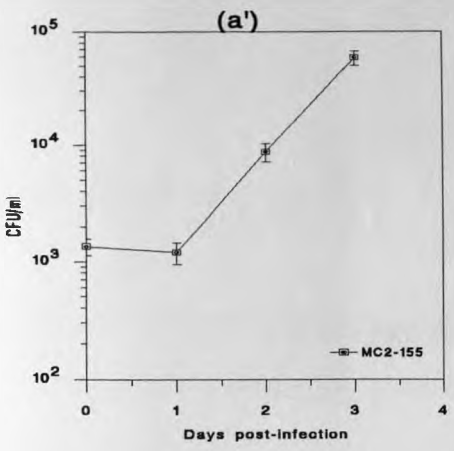
A time course, post-incubation was then followed and culture supernatants from cells infected with each of the strains of *M. smegmatis* were tested for NO. Bacterial viability assessment was then also performed for each strain by CFU determination. The concentration of saponin (0.2% wt/vol final concentration) used in these experiments was shown not to directly influence bacterial growth or viability. Furthermore, no NO_2^- production (< 3nM/ml) was detected in the cell supernatants following infection with any of the strains of *M. smegmatis* in these experiments. The results indicate that human monocyte-derived macrophages fail to control the intracellular growth of any of the different strains of *M. smegmatis* examined [Figures:5.4a-e & a'-e'].

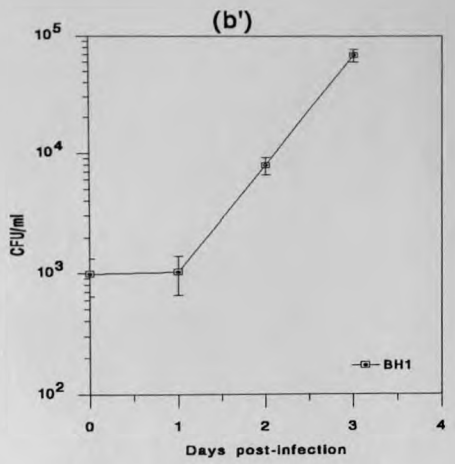
Figure 5.4
Intracellular growth of different strains of M. smegmatis within unactivated human monocyte-derived macrophages

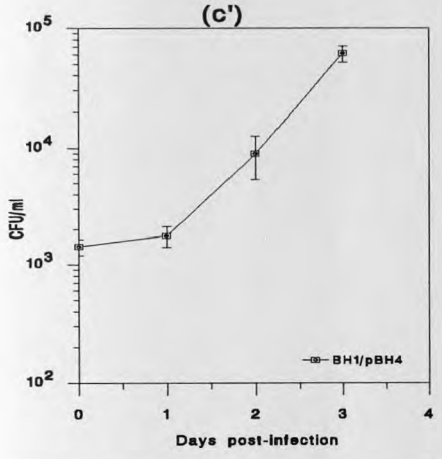
DONOR 1

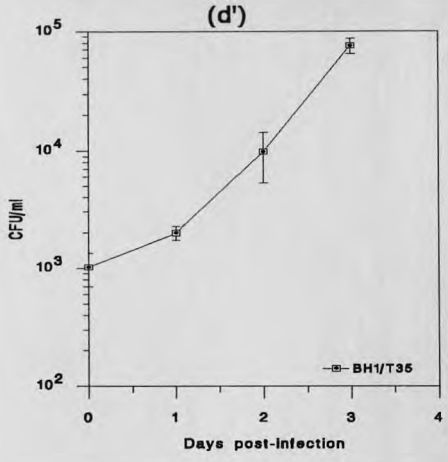


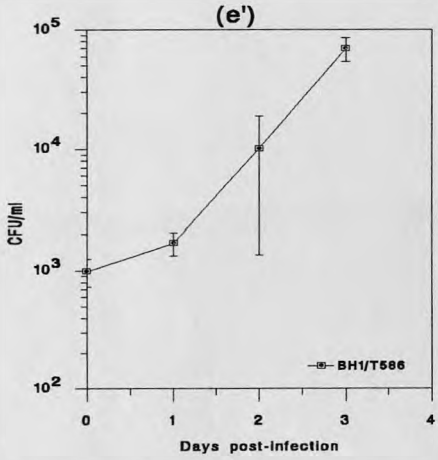
DONOR 2


(a')
CFU/ml
10^5
10^4
10^3
10^2
MC2-155
0
1
2
3
4
Days post-infection


(b')
CFU/ml
10^5
10^4
10^3
10^2
BH1
0
1
2
3
4
Days post-infection


(c')
CFU/ml
10^5
10^4
10^3
10^2
BH1/pBH4
0
1
2
3
4
Days post-infection


(d')
CFU/ml
10^5
10^4
10^3
10^2
BH1/T35
0
1
2
3
4
Days post-infection


(e')
CFU/ml
10^5
10^4
10^3
10^2
BH1/T566
0
1
2
3
4
Days post-infection

Figures 5.4 (a-e & a'-c')

These figures illustrate the intracellular growth of five strains of *M. smegmatis* within non-activated 4-day old human monocyte-derived macrophages obtained from two different donors, donor 1(a-e) and donor 2 (a'-e'). The macrophage cultures were infected with different strains of *M. smegmatis* at a bacteria:cell ratio of 5:1. Extracellular bacteria were removed after 4hrs, and the number of intracellular BCG determined as zerotime (0). Viability counts of bacteria were determined over a time course of 1, 2 and 3 days post-infection, by agar plating on 7H10 agar over 48 hours. Each macrophage donor was tested in three experiments performed at different days. Each data point represents means of nine replicates +/- SD.

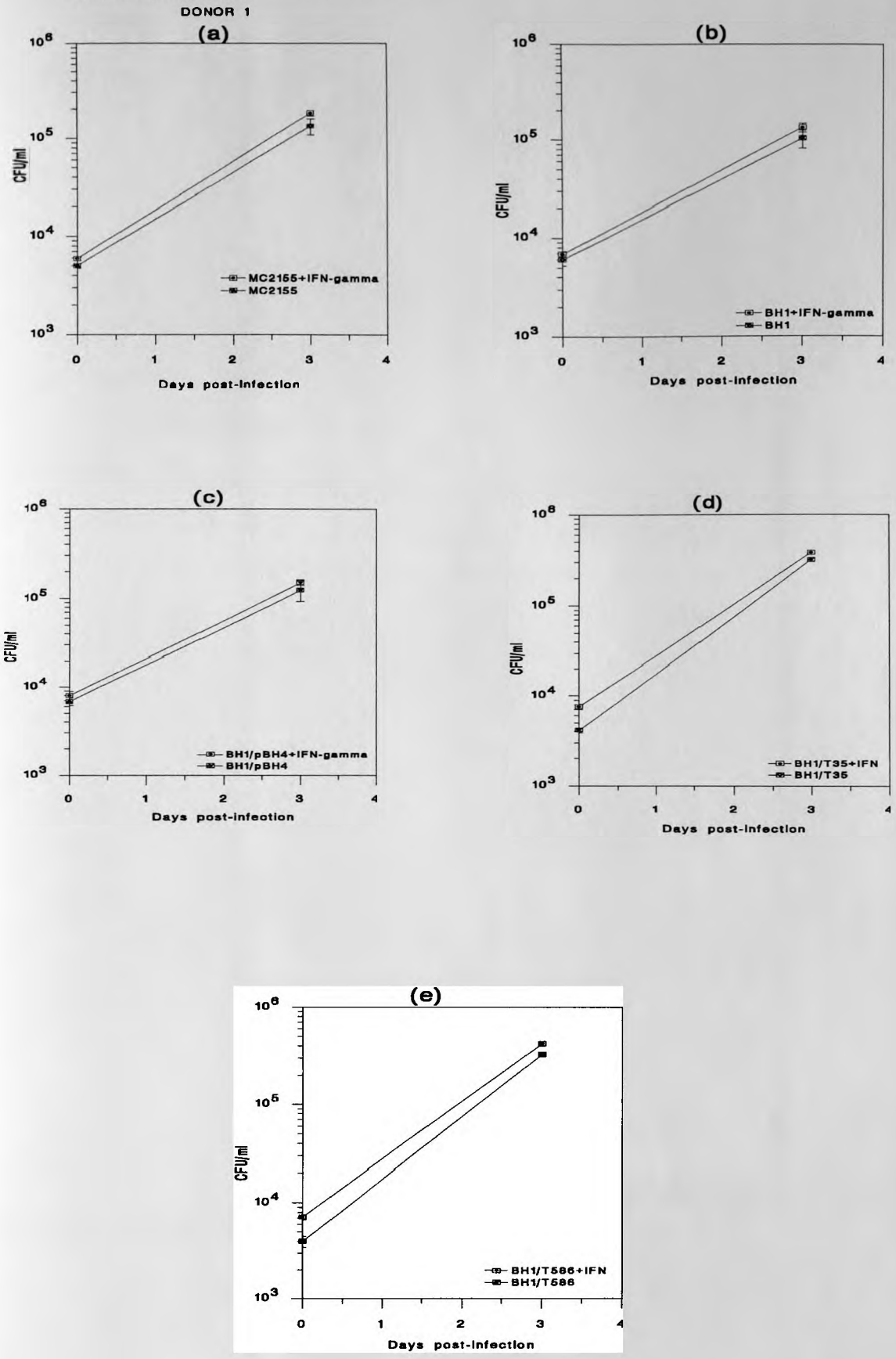
5.3.5 Intracellular growth of different strains of *M. smegmatis* in activated human monocyte-derived macrophages

In a further series of experiments, 4-day old human monocyte-derived macrophages from two donors [Donor 1 & 2] were activated with 1000U/ml of recombinant human IFN-γ for 18hrs and 10ng/ml of LPS for 4hrs prior to infection with 3 different strains of *M. smegmatis* at a bacteria to cell infection ratio of 5:1. Extracellular bacteria were removed and the number of intracellular *M. smegmatis* CFU was then determined at 4hrs post-infection as time zero [0]. The results indicate that activated human macrophages failed to inhibit any of the strains of *M. smegmatis* over a 3 day post-infection period (Figures: 5.5 a-c & a'e'). Moreover, no NO was detected in cell culture supernatants i.e. < 4nM/ml from monocytes cultures stimulated with IFN-γ + LPS or infected with *M. smegmatis* (Appendix 10.3).

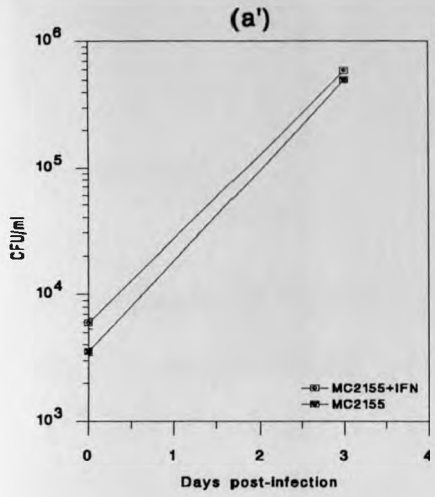
5.3.6 Inhibition of *M. smegmatis* growth within human monocyte-derived macrophages not observed in murine J774 cells.

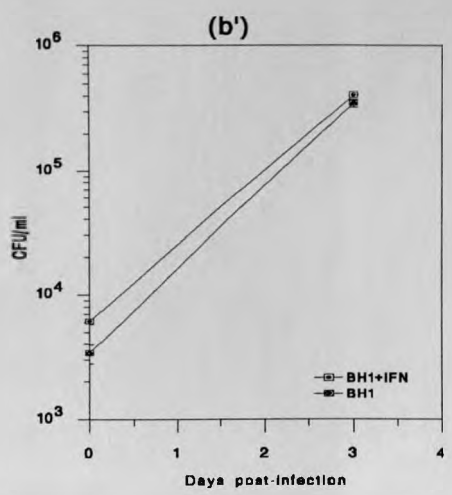
In these experiments activated murine J774 cells and 4-day old human monocyte-derived macrophages were infected with bacteria at an infection ratio of 5:1 in two separate experiments. Both activated and un-activated murine and human cells were used as controls. Extracellular bacteria were not removed. The same number of bacteria were also added to RPMI-1640 media, as a cell-free control. The cell cultures were lysed 24 hrs post-infection with 0.2% saponin, with the same concentration of saponin also added to the medium control wells. The survival/growth of the bacteria was then determined by CFU determination as shown in Table 5.2. Visual assessment of CFU determinations and of colony size indicated that the growth of all 5 strains of *M. smegmatis,* following release from human macrophages by 0.2% saponin lysis, remained inhibited to a degree for the first 24 hrs after plating out, as compared to equivalent numbers of bacteria grown in cell-free cultures as control. This suggested that the bacteria were being inhibited to a degree by their intracellular environment. However this inhibitory effect disappeared after incubating the plates for a further 24 hrs (Table 5.2). Since 0.2% saponin was also added to the medium control wells, this effect could not be accounted for by the effects of saponin alone. No nitrite moreover was detected in human cell

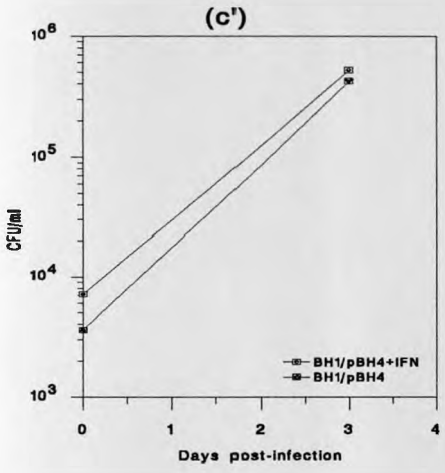
Figure 5.5
Intracellular growth of different strains of M. smegmatis in activated/non-activated human macrophages

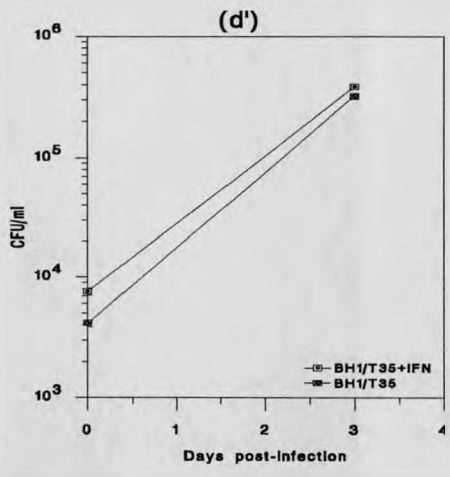


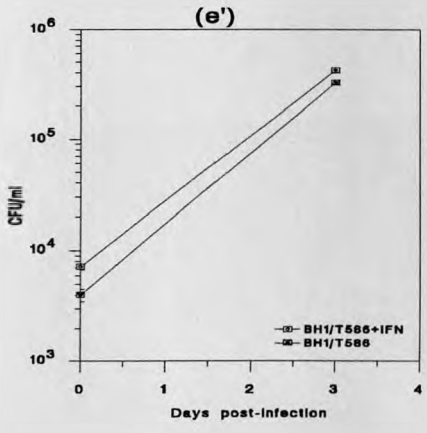
Donor 2


(a')
CFU/ml
Days post-infection
MC2155+IFN
MC2155


(b')
CFU/ml
Days post-infection
BH1+IFN
BH1


(c')
CFU/ml
Days post-infection
BH1/pBH4+IFN
BH1/pBH4


(d')
CFU/ml
Days post-infection
BH1/T35+IFN
BH1/T35


(e')
CFU/ml
Days post-infection
BH1/T586+IFN
BH1/T586

Figures 5.5 (a-e & a'-e')

These figures illustrate intracellular growth of five strains of *M. smegmatis* within activated 4-day old human monocyte-derived macrophages from two human donor sources were pre-activated with IFN-γ (1000U/ml) for 24hrs and LPS (10ng/ml) for 4 hrs, prior to infection with various strains of *M. smegmatis*, at bacteria:cell ratio of 5:1. Unactivated control cell cultures were similarly infected. The number of intracellular *M. smegmatis* CFU was determined as zerotime (0). The viability of bacteria was determined by estimating CFU counts 3 days post-infection. The data in figures (a-e) donor 1, and (a'-e') donor 2 represents mean of triplicates +/-SD < 5% of mean (results not shown).

Table 5.2

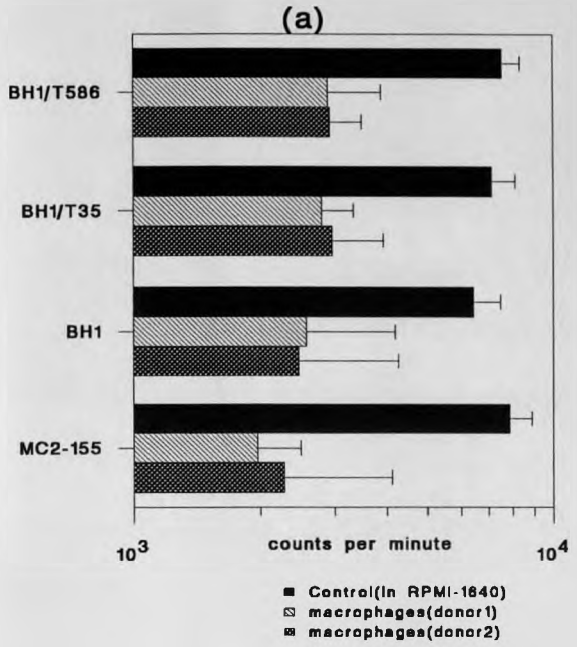
Semi-quantitative assessment of the presence of visible colony forming units (CFU) after release by lysis[a] of infected J774 murine cells and human monocyte-derived macrophages

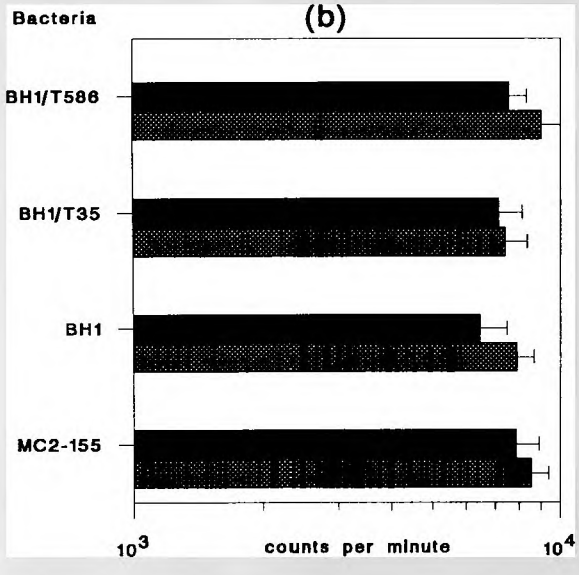
Time (hours)	[b]Bacteria released from macrophages		Bacteria cultured in medium control[c]
	Activated[d]	Un-activated[e]	
J774 murine cells			
24	+++[d]	++	++
48	+++[d]	++	++
Human monocyte-derived macrophages			
24	----	----	++
48	++	++	++

[a] 0.2% saponin used to lyse the infected cell cultures.
[b] 5 different strains of *M. smegmatis.*
[c] Control cultures were grown in RPMI-1640 + 5% serum.
[d]Activated with IFN-γ (500U/ml) plus LPS (10ng/ml) for 4hrs prior to infection for J774 cells, while for human cells IFN-γ (1000U/ml) for 18 hrs and LPS (10ng/ml) 4hrs prior to infection .
[e]Not activated with cytokines.
[d]Depicts presence of bacterial growth at higher density than seen in the control[c] wells.
++ (Presence) and -- (Absence) of CFU's.

This table describes semi-quantitative assessment of CFU following release from infected cells. J774 murine cells and 4-day old human monocyte-derived macrophages were pre-activated with cytokines for 4hrs and then infected with different strains of *M. smegmatis* at an infection ratio of 5 bacteria/cell. Extracellular bacteria were not removed and intracellular bacteria were determined by lysis with 0.2% saponin 24hrs post-infection. The survival/growth of the viable bacteria was determined by plating on 7H10 agar culture medium. Kanamycin was used for all transfected strains. Plates were visualised for presence or absence of CFU's 24 and 48 hours post-incubation.

Figure 5.6
Growth of different strains of M. smegmatis in human macrophages (a) and murine J774 cells (b)





Figures 5.6 (a, b)

The figure represents the metabolic activity of 4 strains of *M. smegmatis* following release from 4-day old human monocyte-derived macrophages from (a) two different human macrophage donor sources, and (b) J774 murine cells, as determined by [^{3}H] uridine incorporation over 4hrs. Both the cell cultures were infected with 5 bacteria/cell. Extracellular bacteria were not washed, and an equal number of bacteria were grown in RPMI-1640 (cell-free media). The bacterial cultures were harvested and counts determined. The data obtained from 3 experiments for each cell culture type, is represented as counts per minute (cpm). Each bar represents mean values of quadruplicates +/- SD.

supernatants in these experiments (Appendix 10.3). The results were also confirmed by radiolabelling the bacteria with [^{3}H] uridine for 4 hrs which revealed that there was a statistically significant reduction $p < 0.5$ in isotope uptake at 24 hrs post-infection [Figure: 5.6a].

In the case of J774 murine cells however visual assessment of CFU and colony size showed that there was no inhibition of growth of any of the strains of *M. smegmatis* following release from saponin lysed cell cultures, in the first 24 hrs after plating on nutrient agar, as compared to the same number of bacteria grown in cell free controls (Table 5.2). This indicates that the bacteria were not being inhibited by their intracellular environment. The results were confirmed by radiolabelling with [^{3}H] uridine for 4 hours as evident in figure 5.6b, where it can be observed that there is no reduction in bacterial uptake of radio-isotope over the first 24 hrs post-infection. It should be noted however that high concentrations of nitrite (Table 5.1) were detected in these murine cell experiments.

5.3.7 The effect of sodium nitrite and sodium nitroprusside on the growth kinetics of different strains of *M. smegmatis in vitro* culture medium.

Graded doses of sodium nitrite ($NaNO_2$) and sodium nitroprusside (SNP) were added to cultures of *M. smegmatis* in 7H9 medium. The survival/growth of bacteria then assessed both by [^{3}H] uridine labelling over 4hrs and by the semi-quantitative assessment of CFU counts at 24 and 48 hrs post-culture. It was found that both compounds had a direct inhibitory affect on the extracellular growth of the different strains of *M. smegmatis* (Tables 5.3 & 5.4). $NaNO_2$ at 25-100 mM was shown to be totally inhibitory (100%) to all the strains of *M. smegmatis* as assessed by [^{3}H] uridine labelling and by visual assessment of CFU growth of *M. smegmatis* (Table 5.3). Moreover at 25mM of $NaNO_2$ no growth was apparent for 48 hrs, however some growth appeared after 48 hrs. Hence the MIC (minimal inhibitory concentration) for sodium nitrite was determined to be 6.2mM for all the strains of *M. smegmatis* examined.

Similarly, SNP (sodium nitroprusside) completely inhibited the growth of all the strains at concentrations of 25-50mM, as determined by semi-quanitiative estimation of the growth/viability of *M. smegmatis* by visualising the number and size of the colonies. However SNP at concentraions as low as 10mM was found

S. No.	$NaNO_2$	mc^2-155	% GI[a]	24 hr.	48 hr.	BH1	% GI[a]	24 hr.	48 hr.	BHI/p BH4	% GI[a]	24 hr.	48 hr.	BH1/ T35	% GI[a]	24 hr.	48 hr.	BH1/ T586	% GI[a]	24 hr.	48 hr.
	(mM)	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]
1	0	51251	0	++	++	40589	0	++	++	42613	0	++	++	47151	0	++	++	46367	0	++	++
2	50	135[d]	100	-	-	126	100	-	-	111	100	-	-	111	100	-	-	125	100	-	-
3	25	120	100	-	-	140	100	-	-	124	100	-	-	80	100	-	-	101	100	-	-
4	12.5	286	100	-	+	161	100	+/-	+/-	215	100	+	+	202	100	+	+	191	100	+	+
5	6.2	19475	62	+	++	3653	91	+/-	+/-	5538	87	+	+	9901	79	+	+	12519	73	+	+
6	3.1	21525	58	++	++	17453	57	++	++	16615	61	++	++	13673	71	++	++	14373	69	++	++
7	1.5	23575	54	++	++	19076	53	++	++	19601	54	++	++	15559	67	++	++	18546	60	++	++
8	0.7	30750	40	++	++	19888	51	++	++	22158	48	++	++	17917	62	++	++	21328	54	++	++
9	0.3	37925	26	++	++	26788	34	++	++	25567	40	++	++	23575	50	++	++	32456	30	++	++
10	0.1	46125	10	++	++	31253	23	++	++	31107	27	++	++	37249	31	++	++	36166	22	++	++
11	0.01	51241	0	++	++	32065	21	++	++	36221	15	++	++	38192	19	++	++	40802	12	++	++
12	0.001	50214	0	++	++	32471	20	++	++	40254	0	++	++	39135	17	++	++	42193	9	++	++
13	0.0001	51248	0	++	++	36530	10	++	++	41523	0	++	++	44793	5	++	++	45896	0	++	++

Table 5.3
The effect of Sodium nitrite on different strains of *M. smegmatis* in 7H9 culture medium.

[a] % Growth inhibition (GI) is calculated by the following equation = [1- counts per minute (cpm) in the presence of $NaNO_2$ / cpm control] x 100.
[b]Counts per minute (cpm) obtained by radiolabelling the proliferating bacteria with [^{3}H] uridine incorporation over 4 hours.
[c]Semiquantitative indication of bacterial growth as determined by visual assessment of colony forming units.
++ (Presence of growth) or -- (Absence of growth) of colony forming units.
+/- depicts presence of bacterial growth at lower density than seen in control wells (without sodium nitrite).
[d]Data represents mean of triplicates obtained from 3 different experiments (sd < 10% of mean, not shown).

This table shows the effect of sodium nitrite on growth kinetics of five different strains of *M. smegmatis*. Graded dilutions of sodium nitrite were prepared, and incubated with 10^5 bacteria/well in 7H9 broth culture. The bacterial cultures were harvested 24 hours post-incubation and survival/growth determined by radiolabelling with [^{3}H] uridine for 4 hours, and plating out on 7H10 agar respectively. The bacterial cultures were then visualised for the presence of CFU's 24 and 48 hours post-incubation as indicated in the above table.

S. No.	SNP	mc²-155	% GI[a]	24hr.	48hr.	BH1	% GI[a]	24hr.	48hr.	BH1/p BH4	% GI[a]	24hr.	48hr.	BH1/ T35	% GI[a]	24hr.	48hr.	BH1/ T586	% GI[a]	24hr.	48hr.
	(mM)	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]	cpm[b]		CFU[c]	CFU[c]
1	0	4836[d]	0	++*	++	4551	0	++	++	4782	0	++	++	2000	0	++	++	1971	0	++	++
2	50	136	100	-	-	126	100	-	-	111	100	-	-	111	100	-	-	125	100	-	-
3	25	112	100	-	-	140	100	-	-	124	100	-	-	80	100	-	-	101	100	-	-
4	10	89	100	-	+	159	100	-	+	215	100	-	+	202	100	-	+	191	100	-	+
5	5	5475	0	++	++	5434	0	++	++	5363	0	++	+	2003	0	++	++	2020	0	++	++
6	2.5	5010	0	++	++	4685	0	++	++	4769	0	++	++	1899	0	++	++	1993	0	++	++
7	1.25	4960	0	++	++	4728	0	++	++	4710	0	++	++	1952	0	++	++	1778	0	++	++
8	6.25	4853	0	++	++	4479	0	++	++	4524	0	++	++	2055	0	++	++	2099	0	++	++
9	3.17	4441	0	++	++	4556	0	++	++	4554	0	++	++	2008	0	++	++	1786	0	++	++
10	1.58	4701	0	++	++	4275	0	++	++	4542	0	++	++	2046	0	++	++	1925	0	++	++
11	0.79	5080	0	++	++	4649	0	++	++	4705	0	++	++	2046	0	++	++	1966	0	++	++

Table 5.4
The effect of Sodium nitroprusside (SNP) on different strains of *M. smegmatis* in 7H9 culture medium

[a] % Growth inhibition (GI) is calculated by the following equation = [1- counts per minute (cpm) in the presence of SNP / cpm control] x 100.
[b]Counts per minute (cpm) obtained by radiolabelling the proliferating bacteria with [^{3}H] uridine incorporation over 4 hours.
[c]Semi-quantitative indication of bacterial growth as determined by visual assessment of colony forming units.
++ (Presence of growth) or -- (Absence of growth) of colony forming units.
+/- depicts presence of bacterial growth at lower density than seen in control wells (without sodium nitroprusside).
[d]Data from 3 different experiments, mean of triplicates (sd < 10% of mean not shown).

This table shows the effect of sodium nitroprusside (SNP) on growth kinetics of five different strains of *M. smegmatis*. Graded dilutions of SNP were prepared, to which 10^5 bacteria/well was added in 7H9 broth culture. The bacteria were washed 24 hours post-incubation and survival/growth determined by radiolabelling with [^{3}H] uridine for 4 hours, and plating out on 7H10 agar. The bacterial cultures were visualised for the presence of CFU's 24 and 48 hours post-incubation as indicated in the table above.

to inhibit the metabolic activity of all the strains by 100%, as assessed by [^{3}H] uridine labelling [Table 5.4]. Furthermore, no colony growth was apparent for 48 hrs in culture, although some growth was visually evident after 48 hrs. The MIC (minimal inhibitory concentration) of SNP for *M. smegmatis* was then determined to be 25mM.

5.3.8 Effect of LNMMA (NO synthase blocker), on intracellular BCG growth within human monocyte-derived macrophages

Four-day old human monocyte-derived macrophages were pre-activated with CPS (Con-A stimulated PBMC supernatant), which was used as a crude source of cytokines to elicit the production of RNI (Reactive Nitrogen Intermediates). Macrophages were pre-activated with CPS for 4 hrs before infection with BCG at a bacteria/cell infection ratio of 5:1. LNMMA (500μM), a NO synthase blocker, was also used to look for an effect on intracellular BCG growth of NO. Studies on murine system have previously shown that LNMMA can reverse the inhibitory effect of NO released from activated cells on bacterial growth. An enhancement in the intracelluar growth of bacteria would then be predicted in LNMMA treated cultures than in control cultures which were not treated with LNMMA. LNNMA was also tested for its direct inhibitory action on BCG, by MIC determination (Appendix 10.5), prior to its use in these growth assays. In this series of experiments however, 10 human donors were examined, including a TB and a CGD patient and eight normal healthy controls. Macrophages from only one normal Caucasian donor source however, produced detectable amounts (9nM/ml) of nitrate as determined by chemiluminescence assay (Table 5.5). In parallel there was also significant change in the bacterial growth (+56%), which refers to increased metabolic activity of dividing mycobacteria as evidenced by [^{3}H] uridine incorporation in the same macrophage donor. Another normal Asian donor however was also shown to exhibit an enhanced metabolic activity (+34%) of intracellular BCG, but failed to produce any nitrite (Table 5.5).

Further to the preliminary results obtained by [^{3}H] uridine incorporation, experiments were extended to confirm those results by performing colony forming unit estimates to actually determine the viability of the bacteria. Macrophages from the donor which produced detectable nitrate levels (Table 5.5), along with 4

Table 5.5

Intracellular BCG growth in 4-day old human monocyte-derived macrophages in the presence/absence of LNMMA as determined by [^{3}H] uridine incorporation.

	Control[a] (- LNMMA)				+ LNMMA (500μM)				
Mϕ donors	Zerotime[b]	Day 3[c]	% Δ[d]	Nitrate(nM/ml)[e]	Zerotime[b]	Day 3[c]	% Δ[d]	Nitrate(nM/ml)[e]	Effect of LNMMA (% Δ[f])
1	2154[g]+/-154	8562+/-548	+297	< 4	2563+/-215	11025+/-1002	+330	<4	+11
2	1985+/-214	6524+/-542	+228	< 4	2154+/-154	7548+/-1254	+250	<4	+9
3	1458+/-98	9154+/-563	+527	< 4	1897+/-78	8451+/-895	+345	<4	-35
4	2415+/-97	10234+/-652	+323	< 4	2145+/-114	11452+/-874	+433	<4	+34*
5	1568+/-254	6854+/-452	+337	9	2458+/-456	15421+/-254	+527	< 4	+56*
6	2254+/-125	12654+/-587	+460	< 4	2154+/-698	13251+/-789	+515	<4	-11
7	2541+/-136	16485+/-589	+548	< 4	2568+/-114	15421+/-998	+500	<4	-9
8	2159+/-145	14568+/-1254	+574	< 4	2458+/-258	13254+/-365	+439	<4	-24
9	2011+/-158	15264+/-1025	+659	< 4	2569+/-548	12541+/-1254	+388	<4	-42
10	1999+/-114	16254+/-1254	+713	< 4	2254+/-225	15487+/-1011	+587	<4	-18
11	2222+/-56	13458+/-1000	+505	< 4	2365+/-114	14857+/-1123	+528	<4	+14

[a]BCG-infected macrophage cultures without addition of LNMMA were used as controls.
[b]The number of intracellular BCG was determined 4 hour post-infection as zerotime (0).
[c]3 days post-infection.
[d]Percentage change in bacterial growth was determined by = [1-cpm at day 3/ cpm at zerotime] x 100.
[e]Nitrate levels in supernatants from control macrophage cultures (-LNMMA) were determined by chemiluminescence at 24 post-infection.
[f]Percentage change in bacterial growth due to LNMMA was obtained by = [1-% Δ (+LNMMA)/ % Δ control (-LNMMA)] x 100.
+ (Growth enhancement), - (Growth inhibition)
[g]Data represent mean of triplicates +/-SD expressed as counts per minute (cpm).
* Statistically significant $p < 0.5$

This table represents the effect of LNMMA, a NO synthase inhibitor, on intracellular BCG growth within 4-day old human macrophages from 11 different macrophage donors. Macrophage cultures were infected with 5 BCG/cell. Intracellular bacteria were determined 4 hr post-infection as zerotime. Cultures were primed with 500 μM LNMMA 4 hour before infection. Infected macrophage cultures were then lysed and harvested 3 days later. The surviving proliferating bacteria were assessed by incorporation of [^{3}H] uridine.

other donors were studied to look at the direct effect of LNMMA, i.e. blocking of RNI (Reactive Nitrogen Intermediates) on intracellular BCG growth. These experiments were performed using a variety of macrophages at different days post culture, i.e. 1, 4, 7, 10, & 14. In these experiments the viability of BCG was determined by microcolony counting as described in section 2.2.5. The results expressed as CFU/ml, as detailed in Tables 5.6 A-E, show that there was no enhancement of intracellular mycobacterial growth following blockade of the cellular RNI system, by LNMMA, either in the donor macrophages which were previously identified as producing detectable amounts of NO, or in non-producing controls. Lack of any effect of LNMMA, i.e. enhancement of the intracellular growth or reversal of mycobactericidal effect as compared to the relevant controls elaborated in tables 5.6 A-E, was evident in all the macrophage cultures, regardless of their age post-culture (Tables 5.6 A-E). Furthermore, no nitrite was detected in the supernatants from infected macrophage cultures in these experiments (Appendix 10.4).

5.3.9 The effect of sodium nitrite ($NaNO_2$) on BCG growth kinetics in 7H9 culture medium

Graded doses of sodium nitrite solution were added to BCG cultures at two dilutions 5×10^5 and 5×10^6 in 7H9 culture medium. After 24 hrs incubation with sodium nitrite, BCG cultures were washed as described in Methods 5.2.5, and the growth of bacteria determined by radiolabelling with [^{3}H] uridine and confirmed by a semi-quantitative visual assessment of colony forming counts [CFU], by inverted microscopy, after 14 days. It was observed that 10mM of ($NaNO_2$) was the minimum concentration required to inhibit radiolabel uptake by the growing mycobacterial cultures, at both dilutions of bacteria tested (Table 5.7). However when colony forming counts (CFU) were visualised, no bacterial colonies were observed at concentrations higher than (25-100mM) of sodium nitrite (Table 5.7). Moreover, even between 1-10mM of sodium nitrite, less colonies were detected in the nitrite-treated plates as compared to controls which were un-treated (Table 5.7).

Table 5.6

Intracellular BCG growth in human monocyte-derived macrophages at different days post-culture and the involvement of Reactive nitrogen intermediates (RNI)

[A] 1-day old Mϕ

				BCG (CFU/ml) x 10^4		
Substances added						
CPS[a]	LNMMA	DNMMA	L-ARGININE	CFU	SD	% Δ[b]
+	+			7.4*	0.16	-26
+		+		12.2	0.26	+23
+	+		+	8.7	0.05	-13
+		+	+	8.7	0.33	-13
1/2 CPS[c]						
+	+			11.7	0.36	+18
+		+		9.3	0.24	-7
+	+		+	7.9	0.15	-21
+		+	+	12.8	0.33	+29
Controls						
control[d]				9.9	0.17	
	+			8.9	0.18	
			+	11.0	0.42	
	+		+	8.7	0.9	
		+	+	9.3	0.14	
+				12.4	0.22	
1/2				7.9	0.16	

No nitrate was detected in the supernatants from these macrophages cultures.

[a] Con-A stimulated PBMC supernatant.
[b]% Growth =[1- CFU in the presence of (x) / CFU control] x 100, (x) = an additive.
[c]1/2 dilution of neat CPS.
[d] BCG- infected monolayers in the absence of any of additives.
*Data represents mean of triplicates +/-sd.

[B] 4-day old Mϕ

Substances added				BCG (CFU/ml) x 10^4		
CPS[a]	LNMMA	DNMMA	L-ARGININE	CFU	SD	% Δ[b]
+	+			6.7*	0.10	-41
+		+		7.3	0.04	-36
+	+		+	7.4	0.09	-35
+		+	+	7.3	0.03	-36
1/2 CPS[c]						
+	+			9.7	0.36	-15
+		+		7.3	0.24	-36
+	+		+	9.1	0.15	-20
+		+	+	9.3	0.33	-18
Controls						
control[d]				11.3	0.35	
	+			10.2	0.10	
			+	8.1	0.13	
	+		+	11.6	0.17	
		+	+	9.3	0.14	
+				10.1	0.09	
1/2				8.7	0.17	

The results summarised in this table are from the macrophage donor which produced detectable levels of nitrite in one of the previous experiments.

[C] 7-day old Mϕ

Substances added				BCG (CFU/ml) x 10^4		
CPS[a]	LNMMA	DNMMA	L-ARGININE	CFU	SD	% Δ[b]
+	+			6.0*	0.01	+50
+		+		7.3	0.02	+82
+	+		+	6.8	0.05	+70
+		+	+	7.0	0.09	+75
Controls						
control[d]				4.0	0.07	
	+		+	2.4	0.02	
		+	+	2.8	0.01	

No nitrate was detected in the supernatants from these macrophages cultures.

[D] 10-day old Mϕ

				BCG (CFU/ml) x 10^4		
Substances added						
CPS[a]	LNMMA	DNMMA	L-ARGININE	CFU	sd	% Δ[b]
+	+			3.5*	0.06	+45
+		+		3.4	0.03	+41
+	+		+	4.7	0.12	+95
+		+	+	1.6	0.03	-34
control[d]				2.4	0.01	

No nitrate was detected in the supernatants from these macrophages cultures.

[E] 14-day old Mϕ

				BCG (CFU/ml) x 10^4		
Substances added						
CPS[a]	LNMMA	DNMMA	L-ARGININE	CFU	sd	% Δ[b]
+	+			5.3*	0.07	+6
+		+		5.2	0.11	+4
+	+		+	3.3	0.07	-34
+		+	+	3.0	0.08	-40
Controls						
control[d]				5.0	0.07	
	+		+	6.2	0.09	
		+	+	2.8	0.01	
+				6.7	0.10	

[a] Con-A stimulated PBMC supernatant.
[b]% Growth =[1- CFU in the presence of (x) / CFU control] x 100, (x) = an additive.
[c]1/2 dilution of neat CPS.
[d] BCG- infected monolayers in the absence of any of additives.
*Data represents mean of triplicates +/-sd.
No nitrate was detected in the supernatants from these macrophages cultures.

These tables [5.6 A-E] illustrate the effect of LNMMA on monocyte-derived human macrophages at different days post-culture. The cell cultures were pre-activated with Conconavalin-A stimulated PBMC supernatant (CPS) neat[a] and 1/2 dilution[b] , added as a macrophage activating cocktail to induce NO release. Human monocyte-derived macrophages were infected with BCG (5 bacteria/macrophage) 3 days post-infection, the infected macrophages cultures were lysed, and the number of viable BCG CFU/cultures was determined as indicated in the table. LNMMA(500μM), a NO synthase inhibitor was used to prevent NO being generated by the activated macrophages. DNMMA(500μM), which does not inhibit NO synthase was used as a control. L-arginine (1mM) was used an agent to reverse the inhibitory effect of LNMMA. Control BCG cultures were maintained in the absence of either of additives.

Table 5.7

Effect of sodium nitrite ($NaNO_2$) on growth kinetics of BCG in culture medium

	counts per minute (3 days)				Visible BCG microcolonies (14 days)	
BCG/ml	**$5x10^5$**		**$5x10^6$**		**$5x10^5$**	**$5x10^6$**
$NaNO_2$(mM)		**% GI [a]**		**% GI [a]**		
0	*3046+/-832		110028+/-3876		++	++
100	ND	ND	86+/-10	99.93	ND	--
50	593+/-218	81	82+/-12	99.93	--	--
25	396+/-107	87	71+/-10	99.94	--	--
10	426+/-189	87	214+/-45	99.81	-/+	-/+
5	831+/-176	73	ND	ND	ND	ND
2.5	838+/-193	73	ND	ND	ND	ND
1	1065+/-189	66	5868+/-391	94.67	-/+	-/+
0.5	2384+/-138	22	ND	ND	++	ND

* Each data point represents mean of triplicates +/- sd obtained from three different experiments.
[a]% Growth inhibition (GI) of bacterial growth is calculated by = [1-cpm experimental / cpm control] x 100
++ (Pesence)or -- (Absence) of bacterial growth.
+/- depicts presence of bacterial growth at lower density than seen in control wells.
ND = not determined.

This table describes the effect of sodium nitrite on BCG growth in culture medium. BCG at two concentrations i.e $5x10^5$ and $5x10^6$ were added to sodium nitrite solution prepared at different dilutions (0.5-100mM) in 7H9 culture medium in 96-well plates. After 24 hours post-incubation with sodium nitrite the number of surviving bacteria were determined by [^{3}H] uridine incorporation and semi-quantitative assessment of bacteria by visualising microcolonies (CFU's) after 14 days.

5.4 Discussion

The major findings of this study, were that activated human blood monocyte-derived macrophages did not produce any nitric oxide as determined by cell supernatant NO_2^- titres but did exhibit a degree of inhibition on the intracellular growth of five *M. smegmatis* strains over the first 24 hrs post-infection. Conversely, the mouse macrophage cell line J774 was shown to produce very high levels of NO following cytokine activation but had no equivalent inhibitory effect on mycobacterial intracellular growth or survival. Five *M. smegmatis* strains with different catalase producing phenotypes, neither exhibited any differential susceptibility to NO production within murine IFN-γ activated cells, nor displayed any differential inhibitory effect on NO production by this cell line. Using *in vitro* NO generating systems, neither BCG nor the five *M. smegmatis* strains were observed to be affected by physiological levels of $NaNO_2$ or SNP. Furthermore, incubation of infected human cells in LNNMA, as a blocker of NOS, failed to affect the intracellular growth of BCG.

The role of nitric oxide (NO) is currently a focus of interest in anti-mycobacterial cellular immunity. Much of the available data on the antibacterial functions of NO however is derived from murine cell systems, where it has been repeatedly shown that cytokine or endotoxin-activated macrophages, generate NO, the production of which has been related to the control of a diverse number of intracellular pathogens including, *Leishmania major, Toxoplasma gondii, Chlamydia trachomatis, Legionella pneumophilia* as well as a variety of Mycobacterial species [Reviewed in Nathan & Hibbs, 1991, Liew & Cox 1991, Nathan, 1992].

The interelationship between the ROI and RNI pathways within cells, especially phagocytes is currently a subject of much interest, as the tide of opinion concerning the relative importance of each in any anti-bacterial mechanism has changed over the last decade, such that current hypothesis now favours a dualistic and possibly synergistic interaction between the two pathways in controlling mycobacterial infections [Hogg *et al*, 1992], although other pathways such as the generation of polyamines are also now being investigated [Andrews, personal communication]. The observation then by Li *et al,* [1992], that catalase inhibited both the induction of NO and NOS when added exogenously to activated murine macrophages, and that this correlated with inhibition of killing of the intracellular parasite *L. major*, was then a potentially very

interesting finding in terms of understanding the interaction between these two pathways. Further studies utilising this model system to test whether the levels of catalase produced by different species of the parasite could account for their differential susceptibility to the host's immune attack however were not feasible, because isolates of Leishmania with the different abilities to produce catalase are not available [Liew personal communication]. An alternative model system i.e. the use of *M. smegmatis* strains was then investigated in this study.

M. smegmatis is a fast growing, avirulent, atypical species of mycobacteria, which is widely utilised by molecular biologists as a convenient vehicle for transfection studies to look at the effects of candidate TB genes on bacterial physiology and pathogenicity. The availability of strains of *M. smegmatis* either transfected with the catalase/peroxidase gene of *M. tuberculosis* [the Kat G gene] or natural mutants with low or no catalase activity were then considered as an ideal model system to study the effects of catalase expression on NO production and consequently on bacterial survival/growth within murine and human macrophages.

The most interesting and suprising finding obtained from this study, was the fact that *M. smegmatis* was observed to grow rapidly within both murine and human macrophages. This was contrary to what was expected, primarily from anecdotal information, and from one paper by Denis *et al,* [1991b] in which *M. smegmatis* was reported to be readily killed by murine peritoneal and splenic macrophages isolated from Bcg^r mice but not from Bcg^s mice over a 48hr period. In our experiments, we utilised the murine cell line J774 for infection studies with *M. smegmatis*, but were unable to demonstrate any similar bactericidal activity.

It is known that cells from different species (i.e. human and murine) and within one species i.e. human leukemic cell lines, bone-marrow, peritoneal, & alveolar etc, can play a critical role determining whether any effect is obtained on inhibition of bacterial growth following cytokine activation [Lowrie, 1990]. Flesch & Kaufmann, [1987] also reported variable and inconsistent effects on BCG growth regulation of murine macrophages obtained from different tissue sites. The J774 cell line utilised in this study was originally isolated from a BALB/c/NIH mouse [Ralph *et al*, 1975], and is known to exhibit a number of characteristics

of peritoneal macrophages in that they exhibit adherent, cytolytic, and phagocytic properties, and express both CR1 and relatively high levels of CR3 complement receptors [Hussman & Johnson, 1992]. Moreover, CR3-mediate phagocytosis has been shown to occur within J774 cells [Hall *et al*, 1991], hence bacteria were always pre-opsonised in our experiments, in fresh human sera, to enhance complement-mediated phagocytosis. The bactericidal effects of Bcg^r mouse peritoneal cells reported by Denis *et al*, (1991b) and the lack of effect of J774 cells cited in this study, may then be attributable to differences in the cell sources utilised, and this would need to be re-examined to resolve this issue. It is of interest however that both Balb/C and NIH mice are known to be Bcg^r phenotypes [Skamene, 1989].

Moreover, of the few studies in which the killing of mycobacteria within murine macrophages has been described, includes, the most frequently cited paper by Chan *et al*, [1992] who, using IFN-γ activated J774 cells, reported a greater than 50% reduction in intracellular BCG levels within a 48 hrs viability assay. This marked reduction however was calculated by measuring [^{3}H] uracil incorporation, which in our hands has not been found to be an accurate index of bacterial survival/growth, but rather an estimate of the metabolic state of dividing bacteria. Furthermore, Flesch & Kaufmann, [1987], using mouse bone marrow derived-macrophages, also showed that the intracellular growth of both BCG and *M. tuberculosis* H37Rv was inhibited by IFN-γ activated cells, although in the same experiment *M. tuberculosis* (Middleburgh strain) was not inhibited. It is also pertinent to note that although RNI's have been previously reported to inhibit the proliferation of a wide variety of intracellular pathogens *in vitro,* including*:* *C. neoformis* [Granger *et al*, 1988], *T. gondii* [Adams *et al,* 1990] *T. musculi* [Vincendeau & Daulouede, 1991], *F. tularensis* [Anthony *et al,* 1992] and *L. monocytogenes* [Gregory *et al,* 1993], these organisms still continued to survive in the presence of RNI.

In vivo studies have also reported that oral adminstration of the L-arginine analogue, MMLA, to BCG-infected mice, in doses that significantly reduced the resulting high urinary nitrate excretion, had no apparent effect on the replication of the mycobacteria [Granger *et al,* 1991]. This treatment however was later reported to block the non-specific protective effects of BCG-infection against an *in vivo* challenge with an unrelated organism *F. tularensis* [Green *et al*, 1992]. Murine macrophages activated *in vitro* with IFN-γ and LPS have also been shown to exhibit arginine-dependent inhibition of a number of intracellular

pathogens including: *Leishmania, Toxoplasma plasmodium, M. leprae, M. avium* and *M. bovis* BCG [Liew & Cox, 1991]. All these anti-parasitic and anti-microbial effects, together with NO production, however could be completely inhibited by L-NMMA [Liew & Cox, 1991]. In this study however, incubation of human cells in a range of L-NMMA concentrations [not all data shown] failed to enhance or inhibit intracellular BCG growth. The data obtained in this study therefore from both human and murine cells, suggest that the protective, i.e. bactericidal function of RNI in host defences to infectious intracellular organisms still remains to be established.

The results cited in this study suggest that differences in catalase expression by the various *M. smegmatis* strains did not effect either, NO production or bacterial survival. This finding then is at odds with the primary rationale for these studies, i.e the results reported by [Li *et al,* 1991], were exogenous catalase was shown to have marked inhibitory effects on murine macrophage NO production and leishmanicidal activity [Li *et al,* 1991]. Lymphokine-induced killing of *M. microti*, by murine macrophages has also been reported to be inhibited by catalase [Walker & Lowrie, 1981], though this effect was not observed with *M. tuberculosis* [Rook *et al*, 1985]. Furthermore, although catalase production by the five strains of *M. smegmatis* utilised in these experiments were not not quantified in this study, catalase expression by these same five strains has been previously determined and published [Cole personal communication]. The lack of any demonstrable effect of the different catalase-expressing strains of *M. smegmatis* on either bacterial intracellular growth or NO production within murine macrophages, supports previous studies in which it was shown that addition of exogenous catalase to murine macrophage monolayers before and after phagocytosis of *M. smegmatis*, did not effect the course of the infection *in vitro* although H_2O_2 release was effectively reduced to basal levels in this system [Denis, 1990].

No differences were observed between the NO_2^- levels in cell supernatants of either murine or human macrophage cultures following infection with either "high" or "low" catalase expressing strains, suggesting that either, such strain differences in expression are too weak to have any real effect on the production of NO generated in response to IFN-γ activation and infection in murine cells, or conversely, that if catalase does play a protective role, the stimuli given in our *in vitro* system may be both non-physiological and too overwhelming to allow expression of any subtle survival advantage to "high" catalase expressing strains.

The latter is not thought to occur as neither "high" nor "low" catalase expressing strains appeared to be inhibited in their growth kinetics to any marked degree by high NO producing activated murine macrophages.

In mice, gamma interferon (IFN-γ), secreted by activated T lymphocytes, is thought to stimulate macrophages to exhibit anti-microbial activities both *in vivo* [Chen *et al*, 1989, Nakane *et al*, 1989] and *in vitro* [Blanchard *et al*, 1991, Douvas *et al*, 1985]. This effect has been associated with the ability of such cells to produce high levels of NO [Chan *et al*, 1992, Denis, 1991b, Flesch & Kaufmann, 1991]. In this study, IFN-γ, was shown to activate the mouse monocytic cell line, J774 to produce significant quantities of NO, similar to those previously reported by Doi *et al*, [1993] and confirmed IFN-γ to be a potent inducer of reactive nitrogen intermediates in mouse macrophages. Furthermore, infection of IFN-γ activated murine macrophages with mycobacteria was found to result in the production of NO, equivalent to that achieved with IFN-γ and LPS in combination. This suggests that mycobacteria themselves can trigger NO release, acting like a second signal to generate NO. This conceivably is a property of the mycobacterial cell wall lipoarabinomannan [LAM]. The pathogens themselves then act as a ' trigger' for the endogenous secretion of NO. However in this study, despite the high levels of NO generated by IFN-γ treated J774 cells, no link was observed between this effect and inhibition of bacterial growth. Indeed *M. smegmatis* was observed to grow more rapidly in IFN-γ activated cells, which would agree more with the findings of Mor *et al*, [1989], who reported an enhancement in the intracellular growth of *M. lepraemurium* in IFN-γ activated mouse peritoneal macrophages. Moreover, other laboratories have reported an inability of rIFN-γ to stimulate murine macrophages to inhibit the growth of or kill virulent strains of intracellular *M. avium* [Bermundez *et al*, 1992, Squires *et al*, 1989].

Similary no effect on either NO production or mycobacterial survival was observed with human PBMC-derived macrophages following activation with CPS, IFN-γ and/or LPS, or following simultaneous infection with either *M. smegmatis* or BCG. This was not so unexpected, as only three reports to date have been published which claim that human phagocytes, under approprtiate conditions can exhibit NO-mediated antimicrobial activity, and in all three instances, the target itself was thought to participate in the inductive process [Denis 1991b, Sherman *et al*, 1991, Munoz *et al*, 1992]. It was therefore not excluded in these

reports, that the terminal oxidases of the organisms themselves, co-cultured with macrophages, rather than the phagocytes, produced the nitrite detected. Other investigators however have reported that cytokine-stimulated human monocyte-macrophages inhibit microbial growth *in vitro* through an L-arginine NO-independent pathway [Cammeron *et al,* 1990, Summersgill *et al,* 1991]. The existence of a cytokine-inducible NO pathway in humans was initially supported by the finding of increased NO_3- levels in the serum and urine of cancer patients undergoing immunotherapy with IL-2 [Hibbs, 1992, Ochoa *et al,* 1992].

Addition of IL-2 to IFN-γ-primed mouse macrophages moreover is also known to trigger NO-mediated tumoricidal activity *in vitro* via stimulation of TNF-α [Cox & Liew, 1992]. Human hepatocytes were also reported to secrete elevated levels of NO_2^-/NO_3^- in response to a cytokine cocktail, which was inhibited by the addition of N^GMMLA [Nussler *et al,* 1992]. Cytosolic NO-synthase from cytokine and LPS-treated human hepatocytes moreover was found to generate NO_2^- in the presence of L-arginine, NADPH, flavin adenine dinucleotide, and tetrahydro-biopterin [Nussler *et al,* 1992]. Furthermore, Martin & Edwards, [1993] recently reported that human monocytes cultured over time can differentiate and produce detectable amounts of NO which are 10-20 fold greater after 10-14 days in culture, than that achieved by freshly stimulated monocytes. However, in the current series of experiments, we could find no evidence of NO production within cell supernatants of human macrophages isolated from two different donors, following IFN-γ activation and/or infection with *M. smegmatis* over a wide range of differentiation states, from 1-day old monocytes to 14-day old mature human macrophages. Such cells moreover were not shown to be mycobactericidal against *M. smegmatis* (not all results shown) although with 4-day old PBMC-derived macrophages we did observe that activated cells did initially inhibit bacterial intracellular growth, over the first 24 hrs, as evidenced by [^{3}H] uridine radiolabelling, but they later failed to control the growth of these non-pathogenic organisms. The series of experiments using cultured macrophages at different stages of maturation moreover, was primarily undertaken to reproduce the experiments of Denis, [1991b], who reported that 14-day old monocyte-derived human macrophages could be activated to synthesise detectable levels of NO_2^- following infection with *M. avium*.

A physiological role for NO in human cells however has been strongly disputed in a recent paper by Schneemann *et al,* [1993], who reported that human macrophages failed to secrete NO after activation with LPS, IFN-γ, GM-CSF, TNF-α, bacteria (heat killed *Listeria monocytogenes & Moraxella catarrhalis*), or proliferating lymphocytes. Furthermore, tetrahydrobiopterin, an essential co-factor of murine NOS was not synthesised by human macrophages nor did exogenous tetrahydrobiopterin support NOS activity in human macrophages. Moreover, no NOS activity was found in cellular subfractions of human macrophages. It then appears that in humans, the inducible high out-put NOS is not conserved as an antimicrobial system of macrophages and the debate then continues. Are human macrophages a source of NO, and does it play any role in infection with intracellular pathogens?

There have been a number of reported studies where the use of *in vitro* generators of NO has been shown to kill various mycobacteria. Chan *et al,* [1992] reported that acidified sodium nitrite used as a chemical generator of NO, was shown to have a strong anti-bacterial effect on *M. tuberculosis* (Erdman) at 5mM, in a cell-free system but that this effect was pH dependent. Killing then only occurred at an acid pH with no direct effect on the bacteria at pH 7. In our studies we also readily demonstrated an inhibitory effect of sodium nitroprusside (SNP) on *M. smegmatis* and of sodium nitrite on both *M. smegmatis* and BCG growth at neutral pH. Neutral pH however was considered to be the more physiological environment of the fully formed phagolysosome, as would be applicable to the later NO production kinetics as compared to the earlier acidic non-fully formed phagolysosome associated kinetics of ROI's. It is of considerable interest to point a report by Crowle *et al,* [1991] who cites convincing evidence that the mycobacteria-containing phagosome is at neutral pH rather than acidic, and therefore challenges the old dogma concerning the intracellular environment that the mycobacteria must face. The higher nitrite levels required to achieve an antibacterial effect in this study however could also be attributed to the known differential susceptibility of different mycobacterial species to NO, described by Doi *et al,* [1993]. Moreover, NO generated chemically by acidification of NO_2^- has previously been reported to show a suppressive effect on cell phagocytosis [Doi *et al,* 1993]. The reduction in the numbers of surviving mycobacteria in studies by Chan *et al,* (1992), might then have been the result of reduced uptake of mycobacteria by the cells.

The lack of any demonstrable effect of the different catalase-expressing strains of *M. smegmatis* on either bacterial intracellular growth or NO production within murine macrophages, supports previous studies in which it was shown that addition of exogenous catalase to murine macrophage monolayers before and after phagocytosis of *M. smegmatis*, did not effect the course of the infection *in vitro,* although H_2O_2 release was effectively reduced to basal levels in this system [Denis *et al,* 1990].

In conclusion, the data in this study would suggest that the generation of NO by IFN-γ activated macrophages is not beneficial for protection against mycobacterial infections.

CHAPTER 6

$CD4^+$ T cytolytic T cells; their role in human mycobacterial immunity

6.1 Introduction

The recent global rise in reported tuberculosis associated with HIV infections has highlighted the importance of the cellular immune system in controlling this important disease. Acquired immunity to intracellular pathogens is thought to rely on antigen-specific CD4+ T-lymphocytes [Kaufmannn *et al,* 1988] which are typically represented as having helper functions, mediating the destruction of intracellular bacteria via cytokine activation of infected macrophages. It is known however that mycobacteria may persist within parasitised macrophages and moreover that interferon-γ activated macrophages have not been convincingly shown to inhibit growth of *Mycobacterium tuberculosis* in humans [Flesch & Kaufmann, 1987, Douvas *et al,* 1985]. Consequently other mechanisms of protection need to be investigated and their antimycobacterial effects examined. Class II restricted T cells have been shown to have cytolytic activity *in vitro* against mycobacterial infected cells [Kumararatne *et al,* 1990, Ottenhoff *et al,* 1988]. The relevance of Class II restricted cytolytic T-lymphocytes (CTL's) in tuberculosis infections however remains controversial with both protective and pathogenic roles being cited in the literature [Kaplan *et al,* 1988, De Libero *et al,* 1988, Pithie *et al,* 1992]. Evidence for the involvement of CTL's in mycobacterial infections *in vivo* at the site of pathology has however been reported for both leprosy [Hancock *et al,* 1989] and tuberculosis [Lorgat *et al,* 1992].

In this study the cytolytic activity of a number of human CD4+ CTL clones was assessed against both DR-matched and mis-matched *M. bovis*-BCG infected macrophages and the consequent effect on mycobacterial viability was determined. Furthermore, the effect on bacterial growth/viability following re-phagocytosis of released BCG, by fresh human monocytes as also examined.

6.2 Materials & Methods

6.2.1 Maintenance of T-cell clones

Two CD4+ mycobacterial antigen-responsive T cell clones, Clone MAW 10.15 (DR-3 restricted) and specific for amino acids 4-13 of *M. leprae* hsp 60; and clone CF 10.23, (DR-4 restricted) which recognises an uncharacterized component of *M. tuberculosis* PPD, were used throughout these experiments. These clones had previously been characterised and shown to have cytolytic activity against MHC-matched antigen-presenting cells [Gaston *et al,* 1990, 1991]. Five other PPD or hsp 65-specific T-cell clones, i.e. CF 92B2.8, CF 92B21.3, CFP 1.2.7, CF B39, CF 6/91/1.6 were also used in subsequent experiments. All T-cell clones were maintained in antibiotic-free medium for at least a week prior to incubation with infected macrophage targets. The clones were used at a minimum of 10 days after restimulation with PHA and feeder cells and 3 days after addition of Interleukin-2 (IL-2). They were maintained by twice weekly feeding with interleukin 2 (IL2) 100U/ml in supplemented antibiotic-free medium, and bi-monthly restimulation using irradiated allogeneic peripheral blood mononuclear cells (PBMC) and PHA.

6.2.2 Cytotoxicity assays

6.2.2.1 Preparation of target cells

PBMC from five Mantoux positive donors were isolated by Ficoll-paque gradient separation and plated out in 96-well round bottomed plate at 5 x 10^5 cells/well in 200µl aliquot of RPMI-1640 medium supplemented with glutamine (2mM) and 10% non-heated fresh autologous serum, [Complete medium (CM)], as described in section 2.2.2. The cells were then dispensed in separate plates for cytotoxicity (plate A) and BCG killing assays (plate B). The monocytes were allowed to adhere to plastic for 4 hrs and the non–adherent cells were removed. Briefly, this involved the gentle re-suspension of the non–adherent cells in their own medium (x 5) and the supernatant then discarded. Fresh media was then added (200µl/well) and the washing procedure repeated (x 2), resulting in an adherent monocyte layer comprising approximately

10% of the original starting population, i.e. $5x10^4$ cells/well. The number of cells/well were counted as described (section 2.2.3). The cells were then finally resuspended in 50µl of media + 10% fresh autologous human serum.

In cytotoxicity assays target monolayers were surface labelled with sodium chromate (^{51}Cr Amersham). Briefly,^{51}Cr, corrected for decay and activity, was added to each well of the target monolayers at a concentration of 2µCi/well in 25µl of RPMI-1640. BCG at a bacteria:cell infection ratio of 5:1 was then added in 20µl aliquots in RPMI-1640 and the plates incubated 16 hrs/37°C/5%CO_2. The supernatant media was then removed by inverting the plates into a shank, and then rapidly drab dried with tissue paper (with care not to let the wells dry out completely). The plates were then washed (x 3) in 150µl aliquots of pre-warmed (37°C) RPMI-1640 + 5% normal human serum (NHS) and the cells finally resuspended in 50µl of RPMI-1640 + 5% NHS.

6.2.2.2 Preparation of effector cells

T-cells clones were harvested, washed once by centrifugation (2000 rpm for 10 minutes), counted and adjusted to give an effector/target ratio of 5:1. The effector T-cells were then added to the BCG infected/non-infected or antigen pulsed or non-pulsed target macrophages in 100µl aliquots as follows:

i) For DR-matched antigen-specific clone-macrophage cytotoxicity assays the following experimental design and controls were incorporated:-

1. Adherent cells + BCG + PPD/peptide (10µg/ml, final concentration)+ T-cells
2. Adherent cells + T-cells
3. Adherent cells + BCG + PPD/peptide
4. Adherent cells alone

ii) *For DR non-matched clone-macrophage Con-A-mediated cytotoxicity assays the following experimental design and controls were incorporated:-*

1. Adherent cells + BCG + T-cells + Con–A (10μg/ml, final concentration)
2. Adherent cells+ BCG + T-cells - Con–A
3. Adherent cells + BCG - T-cells
4. Adherent cells + T-cells
5. Adherent cells alone

The plates were then centrifuged at 500g for 3 minutes and incubated 6 hrs/37°C/5%CO_2, then re-centrifuged at 500g for 3 minutes and the total volume (150μl) of supernatant/well then aspirated into LP-2 tubes (Appleton woods, Birmingham, U.K). Aliquots of 150μl of 1% Triton X-100 (Sigma) was then added to the wells for 0.5h/37°C/5%CO_2 to lyse the remaining macrophage monolayers. Cell homogenates were then transferred to LP-2 tubes for counting using a gamma counter.

The percentage of ^{51}Cr release for each well was then calculated as follows:-

$$\% \text{ Isotope release} = \frac{\text{cpm supernatant}}{\text{cpm supernatant + cpm pellet}} \times 100$$

The level of cytotoxicity [% specific release] in each well was determined as follows:

Cytotoxicity (% specific release) = % isotope release in test well - % isotope release in spontaneous release per well

Results are presented as the mean (SD) of triplicate estimates of the percentage ^{51}Cr release. The SD between triplicates was not normally greater than 5%. Spontaneous release in these assays was usually no greater than 20% of the total uptake of isotope.

6.2.3 BCG growth/inhibition assays

Monocyte or macrophage monolayers were infected with BCG at an infection ratio of 5:1 as described previously (section 2.2.4) and incubated for 18 hrs to allow phagocytosis to occur. Non-phagocytosed BCG were then removed by repeated changing of the supernatant media (x 3), before finally resuspending the infected cell monolayers in 50μl/well of pre-warmed RPMI-1640 + 10% NHS. Either of Peptide/PPD/Con–A at a concentration of 10μg/ml, was then added in 20μl aliquots/well to the respective macrophage targets, and 20μl of CM added to control wells. T cells clones were then added in 100μl aliquots at an effector/target ratio of 5:1 to the target monolayers, and 100μl/well of medium added to target alone control wells. The plates were then centrifuged at 500g for 3 minutes, and incubated for 6 hrs/37°C/5%CO_2. The BCG infected macrophage monolayers were then lysed by adding 20μl/well of 2% saponin (0.2% wt/vol final concentration) for 0.5hr. This concentration of saponin has been shown not to affect viability of BCG.

The following controls were incorporated into each experimental design:-

1. Adherent cells + BCG + T cells + Con–A + (10μg/ml, final concentration)
2. Adherent cells + BCG + T cells - Con–A
3. Adherent cells + BCG - T cells
4. Adherent cells + BCG (To determine % adherence of infected cells)
5. Adherent cells alone (To determine % adherence of un-infected cells)

6.2.3.1 Radiometric labelling assay for determining growth of BCG

To monitor differences in BCG growth following T cell lysis of infected targets, bacterial numbers (per well) were determined by radiolabelling the released bacteria with [^{3}H] uridine as described in section 2.2.7. Briefly, following incubation of effector cells with non-^{51}Cr labelled BCG-infected target macrophages, 100 μl aliquots of the cell supernatants were aspirated into fresh wells of a 96 well U-bottomed microtitre plate and the remaining cells lysed by a combination of resuspension of adherent macrophages using a micro-cell

scraper and the addition of Saponin (0.2% wt/vol, final concentration). The plates were then incubated for 0.5-1 hr/37°C/5%CO_2 to allow complete lysis of the cells, which was monitored by frequent visual observation under an inverted microscope (Olympus, CK2, Japan).

Following lysis, 100µl of 7H9 Middlebrook broth supplemented with 10% ADC enrichment media was added to each well and the contents thoroughly mixed. Aliquots of 50µl of bacteria-cell homogenate were then removed from each well and transferred to a fresh 96 well flat-bottomed microtitre plate for microcolony CFU determination (see below). 2µCi/well of 6-^{3}H uridine (10mCi/ml, specific activity, ICN Flow) in 25µl of RPMI-1640 was then added to the remaining 150µl of cell suspension in each well and to each well containing the aspirated 100µl of supernatant. The plates were then incubated 72 hrs/37° C/5%CO_2 to allow incorporation of the radiolabel into the proliferating bacteria. The bacterial cultures were harvested onto glass fibre filter papers (Printed Filtermat A, 1205-401, Pharmacia Wallac, UK), using an automated cell harvester (Skatron, Finland). The filtermats were air dried and radioactivity assessed using a beta counter (LKB Rackbetta 2). The results expressed are the sum values of the counts per minute (cpm) obtained in both the pellet and supernatant wells for each sample.

6.2.3.2 Determination of BCG viability by colony forming unit estimate by the microcolony counting method

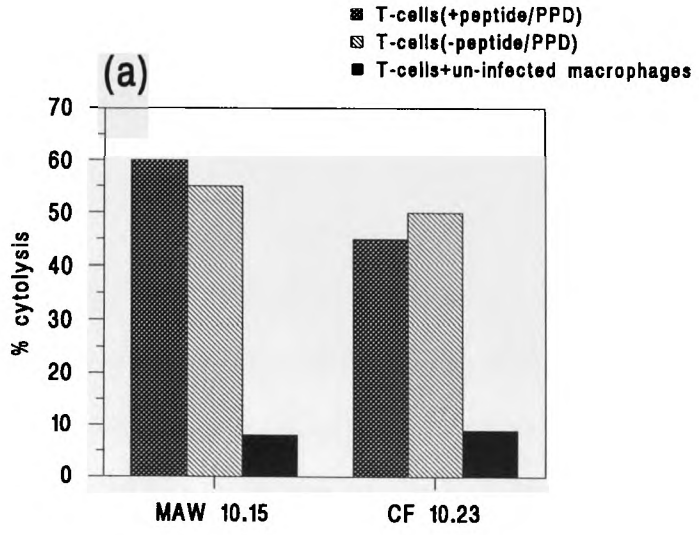
Viable counts of BCG were performed using a microcolony counting technique, the details of which have been described previously (section 2.2.5). Briefly, 50µl aliquots drawn from 200µl of disrupted macrophage lysates were serially diluted (1 in 5) in triplicate in 200µl liquid Middlebrook 7H9 broth (Difco) + 0.2% glycerol + 10% ADC (Difco) supplement in 96-well flat-bottomed microtitre plates. The plates were then incubated 14 days/37°C/5%CO_2 and individual discrete colonies were counted by inverted microscopy (Olympus CK2, Japan). This time was found to be optimal for culture of countable CFUs (20-40 CFU/well) and the colony forming unit concentration /ml of the individual wells was then calculated as follows: CFU/ml = Av x Dil x 20, where: Av = average of counts in replicate wells , Dil = dilution factor of the well row, and (x 20) = correction factor to express bacterial numbers as CFU/ml.

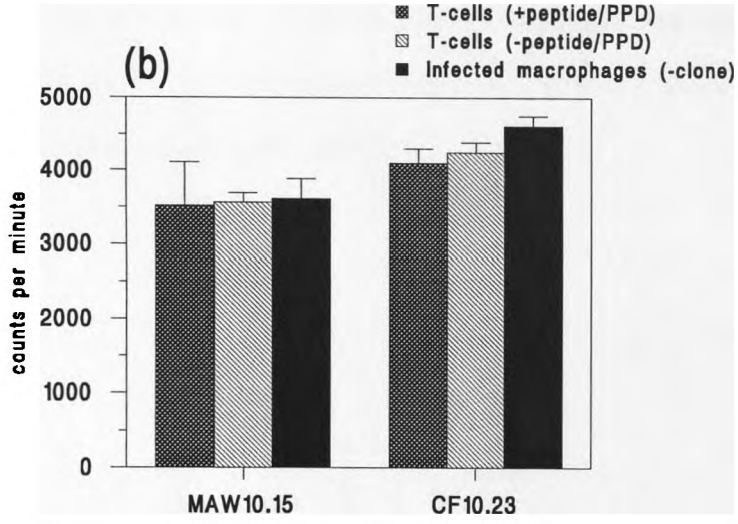
6.3 Results

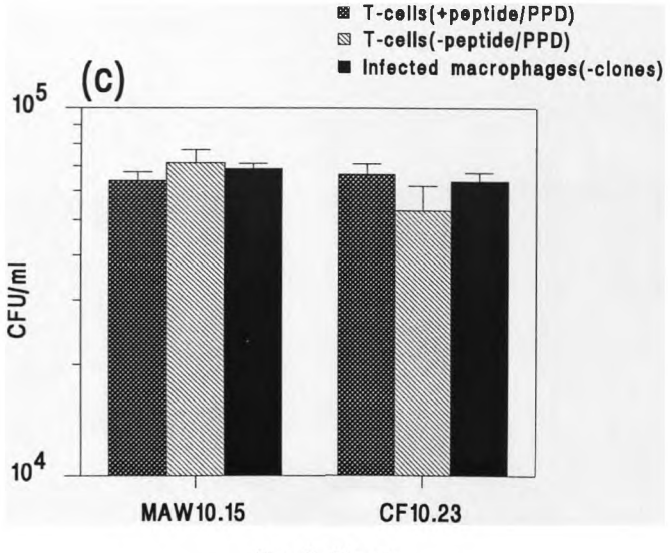
6.3.1 Effect of cytolysis of BCG-infected macrophages by antigen-specific CD4+ T-cells on intracellular bacterial survival

To examine the effect of T cell-mediated lysis of BCG-infected macrophages on bacterial viability, experiments were performed using antigen-specific T cell clones against target macrophages derived from DR-matched donors (Fig 6.1). The survival of released bacteria was then again assessed by both [^{3}H] uridine incorporation and CFU determination (Fig 6.1a & b). The T-cells were co-cultured with the 6-day old BCG-infected, MHC-matched macrophages for 16h at an effector to target ratio of 5:1. Although in principle BCG-infection alone might allow presentation of mycobacterial antigen to these clones additional exogenous specific peptide or PPD were also added at 10μg/ml. Controls consisting of BCG-infected or un-infected macrophage cultures in the presence or absence of antigen were also included. In the presence of specific peptide or PPD for the respective clones marked specific cytolysis (55-60%) of BCG-infected target cells was observed at 16 hours post-incubation (Fig 6.1a). However despite the successful T-cell mediated lysis of the infected macrophages, there was no effect upon the growth or survival of the BCG released, when compared to BCG released from infected macrophages which were not subjected to T cell-mediated lysis. Infection *per se* however did not appear to effect macrophage viability, since (^{51}Cr) release was similar for un-infected (12%+/-5) and infected macrophage target cells (10%+/-7). In addition there was no significant loss of adherent target cell numbers as a result of infection since equivalent levels of (^{51}Cr) incorporation by both infected and un-infected targets were observed (data not shown). Growth and survival were assessed by either [^{3}H] uridine incorporation (Fig 6.1b) or microcolony CFU determination (Fig 6.1c).

Figure 6.1 (a-c)
The effect of cytolysis of BCG-infected macrophages by antigen specific CD4+ T-cells on intracellular survival







T-cell Clones

Figures 6.1(a, b, c)

These figures show the effect of (a) cytolysis of BCG-infected macrophage targets by CD4+ T-cells on intracellular mycobacterial growth/survival, as determined by (b) radiolabelling with [^{3}H] uridine and (c) CFU determination. The T cells were incubated with BCG-infected macrophage targets for 16 hours at an effector to target ratio of 5:1. Cytolysis of DR-matched 6-day old human macrophages was achieved by CD4+ T-cells (MAW 10.15 and CF 10.23) in the presence of 65kd hsp and PPD antigen respectively, as compared to macrophage alone controls in the absence of T-cells. The data in figure (a) represents mean of triplicates of ^{51}Cr release with SD < 5% (data not shown). The effect of cytolysis on intracellular bacterial survival/growth was then assessed both by (b) [^{3}H] uridine incorporation and (c) CFU determination. The data in figures (b & c) represent the mean of triplicates +/- SD (vertical bars).

6.3.2 Effect of Con-A stimulated cytolysis of BCG-infected human macrophages by CD4+ T-cells on intracellular bacterial survival.

To extend these observations, and to circumvent the need for macrophages from MHC-matched donors, T cell clones were induced to lyse target macrophages using the lectin Con-A. A panel of five CD4+ MHC class II restricted, PPD or hsp 65-specific T-cell clones was assessed for cytolytic activity against 6 day old BCG-infected macrophages targets, derived from five different cell donors. Target lysis was determined at 6hr post-infection in the presence or absence of Con A. As illustrated in Table 6.1, the degree of Con A-induced cytolysis at 6hr was found to vary for different clone-macrophage combinations (30-59%), but in all cases was significantly greater than the lysis seen in the absence of lectin which was < 13%. The presence of Con A in the absence of co-culture with T cells however had no discernible affect on isotope incorporation or spontaneous release by infected macrophages.

Having determined that Con-A could be used satisfactorily to obtain MHC-unrestricted lysis of macrophages by T cell clones, assays were performed to assess whether lysis of BCG-infected macrophages had any inhibitory effect on released bacteria. As above, BCG viability and growth were assessed by both [^{3}H] uridine incorporation and microcolony CFU determination (Table 6.1). In this series of experiments, 6-day old macrophage targets were infected for 16 hrs with BCG and then cultured in the presence and absence of effector T-cells for 6 hrs. Although marked macrophage cytolysis (approximately 50%) was achieved after 6hrs culture with T cells in the presence of Con-A, neither [^{3}H] uridine incorporation nor CFU determination revealed any significant difference in the survival or growth of the released bacteria, as compared to those obtained from infected macrophages which had not been subjected to T cell-mediated cytolysis. Indeed in some experiments a modest increase in bacterial metabolism was observed in the presence of T-cell clone and Con-A, as determined by [^{3}H] uridine labelling. No corresponding increase in the number of surviving bacteria however was detected by CFU estimation. Con A itself was shown not to affect either bacterial viability or survival at the concentration used to obtain lectin-mediated cytotoxicity (see appendix 6.1). Thus the larger series of experiments which could be performed by using lectin-mediated

cytotoxicity gave results which were in agreement with those in which antigen-specific cytolysis had occurred, since in both cases cytolysis had no adverse effect on the intracellular bacteria.

Additional experiments were performed to examine the effects of longer periods of co-cultivation of T cells and infected macrophages on bacterial survival. Nine separate experiments were performed using 3 different macrophage donors (Table 6.1). When the duration of the effector-target incubation was increased from 6 to 16 hours (3 experiments), the percentage of Con-A stimulated cytolysis increased on average by approximately 10% above that achieved at 6hrs (i.e. to 40-60%); background release in the absence of Con A remained at < 13%. Although the longer period of co-cultivation achieved consistently higher levels of cytolysis there was again no effect on mycobacterial growth and survival. In a further series of experiments T cell clones were co-cultured with infected macrophages for 48 hrs with similar results (Table 6.1). Cytolysis was not measured at this time point due to the high level of spontaneous lysis of macrophages which occurred over this time.

Experiments were also performed using infected monocytes rather than macrophages as targets of T-cell-mediated cytolysis (Table 6.1). For this purpose 2hr blood-derived monocytes from 3 different donors were utilised. Once more, although substantial cytolysis (35-55%) was readily obtained there was no effect on the subsequent growth of BCG released from the monocytes.

Table 6.1

Bacterial survival following T-cell mediated lysis[1] of BCG-infected macrophages as assessed by [^{3}H] uridine incorporation[2] and CFU determination[3].

			[1] % specific cytolysis[a]		[2] [^{3}H] Uridine incorporation (cpm)							[3] Colony forming units (BCG/ml x 10^{-4})						
Exp.No.	T-cell clones	Mϕ donors	CL+CA[b]	CL-CA[c]	CL+CA	+/-SD	CL-CA	+/-SD	Mϕ[d]	+/-SD	%Δ[e]	CL+CA	+/-SD	CL-CA	+/-SD	Mϕ	+/-SD	%Δ
[A]	6 hrs post-incubation of Mϕ with clones																	
1	CF 92B2.8	A	40	10	9248	534	3500	192	4606	102	+100	3.7	0.2	3.6	0.9	3.8	0.2	-5
2	CF 92B2.8	A	30	5	6157	631	6179	770	5160	862	0	1.0	0.01	1.1	0.04	1.2	0.07	+10
3	CF 92B2.8	B	30	10	2171	80	2128	176	2690	533	0	18.9	1.0	18.4	1.4	14.8	1.1	+4
4	CF 92B2.8	B	35	6	9106	917	10156	634	11154	810	+10	6.9	0.2	3.7	0.2	4.2	0.3	+62*
5	CF 92B2.8	B	30	5	10058	890	9643	120	8741	120	+5	11.2	0.7	9.8	0.9	10.2	1..5	+6
6	CF 92B2.8	D	43	13	8745	452	7854	584	7653	999	+12	0.8	0.05	0.7	0.02	0.8	0.01	+7
7	CF 92B2.8	E	47	13	8745	124	8245	421	8653	124	+1	2.3	0.2	2.1	0.1	2.1	0.2	+5
8	CFP 1.2.7	C	40	12	9152	605	14297	128	12571	450	-15	5.5	0.1	4.8	0.1	5.1	0.1	+3
9	CFP 1.2.7	C	45	10	3470	160	3810	850	3850	896	-8	1.1	0.2	1.4	0.2	1.2	0.2	-7
10	CFP 1.2.7	C	42	9	6077	230	5825	275	5715	280	+6	3.4	0.3	3.1	0.1	3.4	0.3	0
11	CFP 1.2.7	D	50	14	5790	114	9860	1250	4670	1320	0	11.1	0.9	11.7	1.4	11.7	0.9	0
12	CFP 1.2.7	D	47	12	6981	541	5987	256	5999	989	+15	1.0	0.4	0.8	0.01	1.0	0.2	-6
13	CFP 1.2.7	E	39	8	2896	542	2789	541	2985	554	0	1.5	0.05	1.6	0.03	1.4	0.02	+9
14	CF 10.23	C	37	6	3410	470	2810	350	3340	750	+2	2.6	0.4	2.5	0.2	4.4	0.1	-2
15	CF 92B1.3	A	30	6	8754	1252	7895	562	8647	854	+1	3.3	0.1	2.5	0.4	3.3	0.2	+1
16	MAW 10.15	A	35	5	7111	814	6134	730	7129	660	0	5.8	0.2	6.4	0.5	4.2	0.1	+13
[B]	16 hrs post-incubation of Mϕ with clones																	
1	CF 92B2.8	E	55	20	2872	470	2605	214	2616	59	+9	2.7	0.3	2.5	0.1	2.0	0.2	+11
2	CF 92B2.8	D	50	15	2657	217	2340	550	2612	241	+1	13.6	0.2	8.1	0.3	3.9	0.3	+142*
3	CFP 1.2.7	A	40	10	1618	148	1509	204	1633	186	0	2.6	0.2	2.7	0.3	2.6	0.3	0
4	CFP 1.2.7	E	48	8	3230	729	2313	323	2440	415	+32	6.3	0.3	4.3	0.3	3.7	0.1	+56*
5	CF 10.23	D	40	20	1515	86	1639	179	1676	300	0	3.3	0.1	2.2	0.2	2.1	0.1	0
6	CF 10.23	A	60	15	1645	78	1341	298	1294	38	+24	3.6	0.3	3.9	0.3	2.2	0.2	0
7	CF 10.23	A	55	12	1421	258	1232	226	1577	14	0	0.9	0.01	0.7	0.01	0.9	0.09	0
8	CF 10.23	E	60	10	2231	197	2016	116	2696	134	-7	4.0	0.2	4.1	0.2	2.3	0.2	+54*
9	MAW 10.15	B	38	8	2896	421	2754	186	2568	156	+5	3.8	0.3	3.7	0.2	3.5	0.3	+3
[C]	48 hrs post-incubation of Mϕ with clones																	
1	CF 92B2.8	A	35	7	ND	ND	ND	ND	ND	ND	ND	5.9	1.0	6.2	1.4	4.7	0.5	0
2	CF 92B2.8	E	35	5	ND	ND	ND	ND	ND	ND	ND	12.0	2	1.07	1.4	11.7	2	+2
3	CFP 1.2.7	A	40	12	ND	ND	ND	ND	ND	ND	ND	11.4	0.7	10.0	1.0	6.7	1.4	+22*
4	CFP 1.2.7	D	40	14	ND	ND	ND	ND	ND	ND	ND	8.2	1.3	10.2	0.6	7.4	0.6	+29*
5	CFP 1.2.7	D	50	8	ND	ND	ND	ND	ND	ND	ND	10.8	1.6	8.0	1.4	8.3	1.5	0
6	CFP 1.2.7	E	47	11	ND	ND	ND	ND	ND	ND	ND	7.5	1.6	7.0	2	7.2	2	+4
[D]	16 hrs post-incubation of BCG-infected monocytes with clones																	
1	CF 92B2.8	A	50	20	9248	534	4606	1294	10340	989	0	2.6	0.05	2.4	0.09	2.1	0.2	+9
2	CFP 1.2.7	C	55	15	9152	605	14297	2451	11573	1254	-21	5.8	0.2	5.1	0.3	6.0	0.2	0
3	CFP 1.2.7	D	50	20	5790	114	9860	1325	4670	1120	+23	11.2	1.1	11.7	0.9	11.7	1.5	0
4	CF 92B1.3	A	35	16	26906	1252	25945	9458	33340	4158	-2	8.7	0.2	8.0	0.5	7.9	1.0	+9

[a]Percentage cytolysis as a measure of ^{51}Cr release 6 hrs post-incubation of T-cell clones with BCG-infected macrophage targets as given in the methods.

% cytolysis of Mϕ was < 2 in all the above experiments (data not shown), [e] % Δ Percentage change in bacterial growth is calculated = [1- cpm or CFU experimental / cpm or CFU control] x 100.

[b]CL+CA = T-cell clones added to BCG-infected Mϕ in the presence of Con-A,[c]CL-CA = T-cell clones added to BCG-infected Mϕ in the absence of Con-A

[d] Mϕ = BCG-infected macrophages in the absence of T-cell clones.+ (Growth enhancement), --(Growth inhibition).* Statistically significant $p < 0.5$.

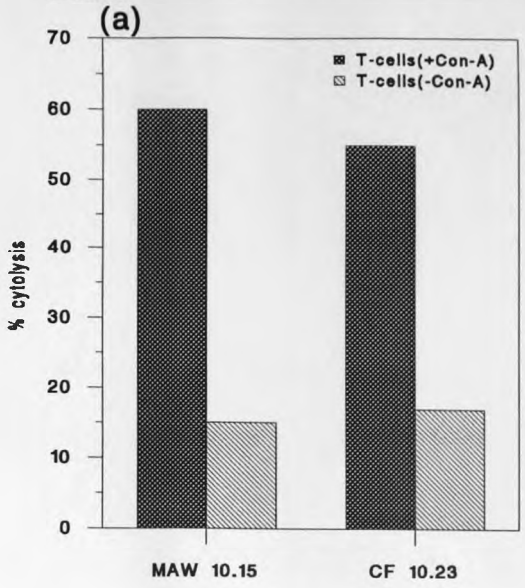
6.3.3 Effect of CD4+ T-cell-mediated lysis of IFN-γ activated, BCG-infected human macrophages on BCG survival.

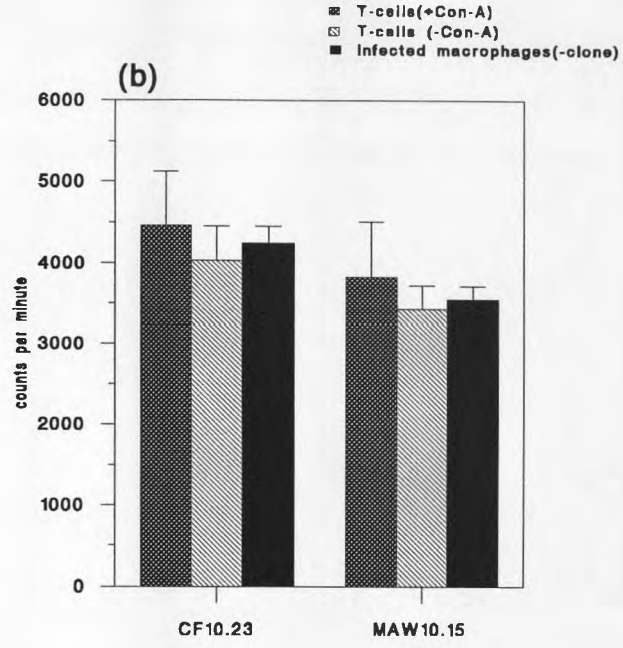
The possibility that the combination of macrophage activation with interferon gamma and T-cell-mediated cytolysis might act synergistically to promote the inhibition or killing of intracellular bacteria was investigated (Fig 6.2 a-c). BCG-infected 6-day old human macrophages were pre-activated with 1000U/ml of human recombinant IFN-γ for 24 hours prior to addition of cloned T cells. Macrophages from two donors were tested using two different T-cell clones. Untreated macrophages were included in each experiment as controls. Figure 6.2 shows that pre treatment of macrophages with IFN-γ did not result in an inhibitory effect on growth or survival of BCG released, following lysis of the infected macrophages by T cells. Treatment of BCG-infected macrophages with IFN-γ alone without effecting T cell-mediated lysis also had no effect on BCG survival.

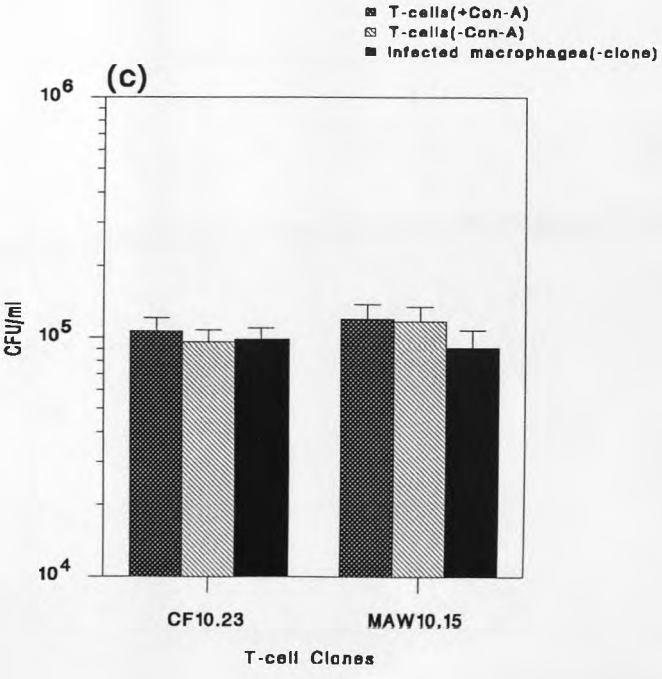
6.3.4 Effect on BCG survival/growth by cytolysis of infected macrophages by CD4+ T-cells and re-phagocytosis of released bacteria by fresh monocyte cultures

It has been suggested that in *in vivo* bacteria released through T-cell mediated lysis of infected macrophages, whose phagocytic abilities may be compromised, are re-phagocytosed and killed by freshly emigrating, activated, monocytes. We attempted to simulate this scenario *in vitro* by adding BCG released from T-cells lysed infected macrophages to 2 hr-old fresh monocyte monolayers for an additional 16 hours. The viability and growth of intracellular BCG within these monocyte cultures was then assessed. Four experiments were performed using three DR-non-matched human macrophage donor targets, and two T-cell clones, i.e. CF B39 and CF 6/91/1.6. Con-A mediated cytolysis of 60-70% was achieved using these clone/target pairings (Figure 6.3a). The BCG released into the cell supernatant following clone-mediated cytolysis of infected macrophages was added to fresh monocyte monolayers and incubated for 16 hours.

Figure 6.2 (a-c)
The effect of CD4+ T-cell-mediated lysis of IFN-gamma activated, BCG-infected human macrophages on BCG survival





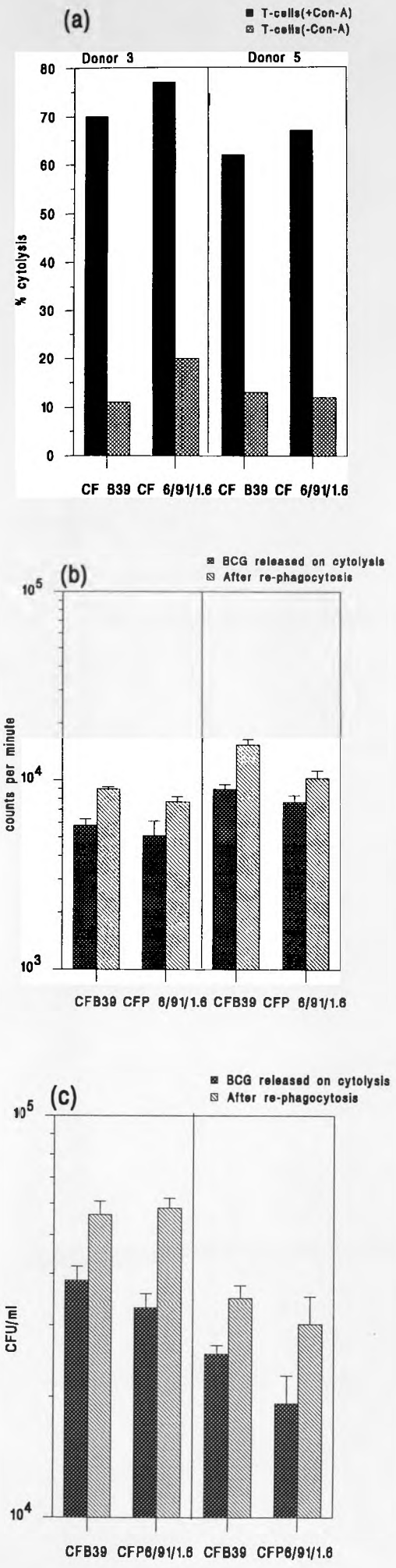


Figures 6.2 (a, b, c)

These figures show the effect of (a) cytolysis of BCG-infected, IFN-γ-activated, DR-non-matched macrophage targets by CD4+ T-cells on intracellular mycobacterial growth/survival, as determined by (b) radiolabelling with [^{3}H] uridine and (c) CFU determination. Cytolysis of 6-day old human macrophages by two mycobacterial-specific CD4+ T-cell clones (CF 10.23 & MAW 10.15) in the presence or absence of Con-A (10μg/ml) at a clone to target ratio of 5:1, as compared to macrophage alone control in the absence of T-cells. The macrophage targets were primed with IFN-γ 1000U/ml for 16 hours prior to infection with BCG and ^{51}Cr release determined at 16hrs post-incubation is shown as percentage cytolysis. The data in fig (a) represents mean of triplicates of ^{51}Cr release with SD < 5% (data not shown). The effect of cytolysis on intracellular bacterial survival/growth was then assessed both by (b) [^{3}H] uridine incorporation and (c) CFU determination. The data in figures (b & c) represent mean of triplicate values +/- SD (vertical bars).

The growth/viability of intracellular BCG within the monocyte monolayers was then assessed by both, radiolabelling by [^{3}H] uridine (Figure 6.3b) and CFU estimate by the microcolony method (Figure 6.3c) following cell lysis with 0.2% saponin. The results show that there was no demonstrable inhibitory or mycobactericidal effect on BCG following re-phagocytosis by fresh monocytes. Rather, BCG released from macrophage cultures continued to grew even after addition to fresh monocytes in all the four experiments (Figure:6.3 a-c).

Figure 6.3
The effect of re-phagocytosis of BCG released on T-cell-mediated cytolysis of human macrophages



Figures 6.3 (a, b, c)

These figures show the effect of re-phagocytosis of BCG following release of BCG-infected macrophage targets by CD4+ T-cells on intracellular mycobacterial growth/survival, as determined by (b) radiolabelling with [^{3}H] uridine and (c) CFU determination. Con-A mediated cytolysis of DR-non-matched, 6-day old human macrophages was achieved by CD4+ T-cells, at an effector to target ratio of 5:1. The data in fig (a) represents percentage ^{51}Cr release determined at 16 hours post-incubation with T cells. Means of triplicate values are depicted (SD < 5% of mean, not shown). The effect on bacterial growth/survival following re-phagocytosis of BCG released by fresh monocyte cultures was then determined at 16hrs post-incubation by (b) [^{3}H] uridine incorporation and (c) colony forming unit estimate. Each column in figures (b & c) represents mean of triplicates +/- SD (vertical bars).

6.4 Discussion

The role of CD4+ cells in acquired resistance against tuberculosis is well established [Orme *et al,* 1993] but has been previously ascribed to their ability to bring about cytokine-mediated activation of macrophages. Current hypotheses however, supported by murine and human studies, would suggest that both cytolytic CD4+ and CD8+ T cells are important in protection against mycobacterial infections [Kaufmann, 1988]. In a number of seminal *in vitro* experiments Rook *et al,* (1985) showed that co-incubation of murine antigen-specific CTL with *M. bovis*-BCG infected macrophages resulted in both macrophage lysis and inhibition of mycobacterial growth. Moreover in our laboratory, we have previously reported that mycobacterial-specific CD4+ T-cells, generated from the blood of BCG-vaccinated subjects and tuberculosis patients, can lyse autologous mycobacterial-infected macrophages [Kumararatne *et al,* 1991, Pithie *et al,* 1992]. In patients with tuberculosis however, the level of cytolytic activity demonstrated by peripheral blood derived CD4+ T cells was found to correlate with the presence of pulmonary cavitation or tuberculosis abscess formation [Pithie *et al,* 1992] suggesting that CTL may contribute more to the tissue damage observed in TB patients than to protective anti-mycobacterial immunity.

The results reported in this study also demonstrate that although CD4+ T cell clones could elicit significant antigen-specific or lectin-mediated cytolysis of BCG-infected macrophages, there was no detectable reduction in survival or growth of the phagocytosed intracellular bacteria. Both radiolabelling and CFU determination methods of assessing bacterial survival and growth were employed because each determined a different parameter of bacterial viability. Radiolabelling with [^{3}H] uridine shows the metabolic state of actively dividing bacteria over a short time course whereas CFU determination depicts actual bacterial survival and growth. The combined results of both assay methods indicate that CD4+ T cell-mediated lysis of infected macrophages neither alters the metabolic state of phagocytosed bacteria nor reduces the viability of the bacteria It is pertinent to these results that in the only previously reported example of the inhibition of bacterial viability by cytolytic T cells, using a murine *in vitro* model system [Kaufmann & Flesch, 1987] bacterial viability was assessed only by [^{3}H] uracil incorporation [Kaufmann & Flesch, 1987]. The results reported in this paper would be more consistent with bacteriostasis rather than killing of bacteria.

In some experiments the presence of cytolytic T cell clones actually enhanced BCG growth, this could be explained if the accumulated products of lysed cells provide a nutrient-enriched environment beneficial to bacterial proliferation. This idea is in contrast to previous reports in which it has been claimed that toxic products are released on cell lysis, and that these may contribute to the extracellular killing of Mycobacteria that occurs in granuloma lesions [Dubos, 1955]. Likewise accumulation of dead polymorphs in TB granulomata was proposed to produce such an effect in infected mice [Silva *et al,* 1989]. However, inhibition of bacterial growth in granuloma tissue may be more dependent upon the deprivation of oxygen to bacteria which occurs in such sites rather than to any build up of toxic cellular products. The finding that rupture of granulomata into oxygen rich sites such as the pleural cavity or alveolar spaces results in explosive bacterial growth is consistent with this, and in the presence of oxygen the growth may actually be fuelled by granuloma-derived cellular debris.

Overall our data support the contention that cytolytic CD4+ T cells may be important mediators of immunopathology in mycobacterial infections, and their generation may be deleterious to the host response rather than contributing to protective immunity. The relationship between protective immunity and the cell-mediated, delayed-type hypersensitivity (DTH) response to mycobacterial antigens has long been debated; some have argued that they are inseparable parts of the same process [Mackaness, 1964] whereas others have suggested that they are essentially separate phenomena [Rich, 1951]. CD4+ T cells can readily destroy infected macrophages, and in so doing could contribute to the tissue necrosis characteristic of "Koch" type hypersensitivity. However our results show that this process fails to kill or inhibit intracellular BCG, suggesting that there may indeed be important differences between the effector mechanisms causing DTH and those which underlie protective immunity, these merit further investigation. It may be pertinent that vaccination with certain non-pathogenic Mycobacteria such as *M. vaccae* is known to elicit a strong protective immunity without evoking a DTH or skin tuberculin hypersensitivity response [Stanford *et al,* 1990, Stanford & Grange, 1993]. Using our experimental protocol, it may then be possible to identify the protective effector cells generated in response to infection with such Mycobacteria, and to identify the mechanistic differences between the two kinds of effector T cells.

The findings confirm similar results obtained this laboratory using polyclonal T cells lines specific for PPD (predominantly CD4+). MHC class II-restricted cytolysis of infected macrophages was observed but these was no effect on the viability of the intracellular bacteria [Pithie *et al,* submitted]. Similarly, Molloy *et al,* [1993] reported that effective lysis of BCG-infected human monocytes by IL-2 generated lymphokine activated killer cells (LAK cells) had no detrimental effect on bacterial viability, but resulted only in the release of viable organisms. *In vivo* however, where host-bacterial interaction is focused in the micro environment of the granuloma, lysis by CTL's of infected macrophages which are incompetent to kill intracellular bacteria may have a beneficial effect by rendering such released bacteria vulnerable to destruction by freshly migrating, cytokine-activated monocytes, as originally expounded by Kaufmann, [1988].

Our experiments showed that cytolysis of infected peripheral blood-derived monocytes which were not allowed to mature into macrophages *in vitro* showed the same lack of effect on the bacteria as was seen when macrophages were used. However, experiments where bacteria were exposed to re-phagocytosis by fresh monocytes following release by CTL-mediated lysis, also failed to check the grwoth of mycobacteria.

Since protective immunity to Mycobacteria is likely to require several effector mechanisms working together, we tested for the possibility of synergy between IFN-γ activated macrophages and T cell-mediated cytolysis. Interferon-γ is known to be a potent activator of murine macrophages, promoting killing of intracellular Mycobacteria [Rook *et al,* 1986c, Walker & Lowrie, 1981, Flesch & Kaufmann, 1988, Khor *et al,* 1986]. In human cells however the reverse effect has been reported, with IFN-γ enhancing intracellular mycobacterial growth [Douvas *et al,* 1985, Rook *et al,* 1986c]. In this study, no anti-bacterial synergy was observed with CTL's and IFN-γ but this may not be surprising since no cytokine has yet been shown to be effective in activating human macrophages to kill *M. tuberculosis*; this is in clear contrast to the important role of IFN-γ in the mouse. Data is emerging however that inhibitory cytokines such as TGF-β1 and IL-10 may also be important in modulating the priming effects of IFN-γ on macrophage antibacterial activity, via down regulation of TNF-α and IL-12 production [Bermudez, 1993, Hsieh *et al,* 1993]. Experiments are then currently in progress to assess the levels of induction of both positive [TNF-α, IFN-γ & IL-12] and

negative [TGF-β1 & IL-10] cytokines in human and murine macrophages following BCG infection, to assess this hypothesis.

Although CD4+ cytolytic T cells do not appear to have a role in bacteriostasis, it is possible that cytolysis by CD8+ T-cells might have such an effect. In mice both CD4+ and CD8+ lymphocytes with cytolytic activity have been demonstrated in experimental models of tuberculosis, leprosy and listeriosis [Kaufmann, 1988]. Interest in CD8+ T cells was recently revived with the provocative finding in β_2-micro globulin "Knock-out" mice, which are unable to generate CD8+ T cells, succumb to progressive disseminated disease when given a normally sublethal dose of *M. tuberculosis* [Flynn *et al,* 1992]. Moreover, provisional results from our laboratory have shown that CD8+ T cells are preferentially generated from human PBMC stimulated with Mycobacteria-infected macrophages, whereas CD4+ T cells are elicited in response to mycobacterial antigens [Panchalingham personal communication]. These CD8+ T cells are likely to be heterogeneous and to include some expressing the γδ T cell receptor [Kronenberg, 1994]. Mycobacteria-specific CD8+ T cells expressing either γδ and αβ TCR will be tested in the protocol which we have described in this paper to determine whether they contribute to antibacterial immunity.

The immune mechanisms of resistance to mycobacterial infection appear to be more complex in humans than in mice and have proved difficult to reproduce *in vitro*. It is probable that multiple mechanisms pathways lead to the final expression of acquired resistance to TB in man. Progress in unravelling such complex interactions may require similarly complex experimental systems, such as organ cultures to reproduce the granuloma environment, or the use of SCID or T cell deficient mice reconstituted with human cells. The latter could allow study of granuloma formation by human effector cells in response to mycobacterial infection *in vivo*. Many laboratories including our own have shown that human macrophages do have an innate potential to check the intracellular growth of Mycobacteria. Further research is required to identify the way in which this occurs, the involvement of T cell mediated immunity in enhancing this property, and how it may be manipulated as a therapeutic strategy.

CHAPTER 7

Comparison of TNF-α production and intracellular mycobacterial growth rates in monocyte-derived macrophages isolated from multicase TB families.

7.1 Introduction

Following inhalation in normal individuals with innate macrophage-based resistance to tuberculosis, mycobacteria are rapidly destroyed before sufficient antigen is generated to sensitise T cells. If this initial inhibition process is overcome, intramacrophage bacterial multiplication is followed by the activation of T cell-mediated immunity [Kumararatne *et al,* 1992]. A single autosomal dominant gene however is thought to govern T-cell independent, macrophage-based resistance to mycobacterial infection in mice and recent genetic analysis indicates the existence of a homologous region on the human second chromosome 2 [Blackwell, 1989]. It has been shown that macrophages of BCG resistant [Bcg^r] mice can be activated earlier and to a higher degree by infection with intracellular bacteria *in vivo* or on exposure to bacterial products *in vitro* [Skamene, 1989]. Furthermore, purified cell wall products [Lipoarabinomannans] from virulent and avirulent strains of mycobacteria are known to differ in their potential to stimulate macrophages to produce TNF in BCG resistant and susceptible strains of mice [Roach *et al,* 1993] and in human cell cultures [Lammas personal communication].

Furthermore, human population studies have also provided evidence that resistance to tuberculosis in man is genetically controlled, and that the basis of this difference may lie in an impaired capacity of macrophages to kill intracellular tubercle bacilli [Crowle & Elkins, 1990, Stead *et al,* 1990]. This innate genetic effect has then been proposed to contribute to the higher incidence of TB observed in certain rational groups [Stead *et al,* 1990].

A number of TB patients who have well documented recurrent infections despite compliance with standard antituberculous drug therapy, have been identified in the UK. Infected individuals moreover are found in several generations, suggesting a possible stable inheritance of susceptibility to this disease.

The hypothesis then, forming the rational for this series of experiments described in this chapter, is that mycobacterial-specific immune responses mounted by infected individuals with a history of recurrent TB infection, from such multicase families are qualitatively and quantitatively different from those of their non-infected siblings and from infected normal individuals. Such patients are then thought to be genetically susceptible to infection, hence were considered as good subjects to look for the expression of genetic markers for susceptibility. Moreover, by identifying the cellular dysfunction in these individuals, such studies may help to characterise the functional effects regulated by such genes.

To investigate this theory, 10 multi-case TB families encompassing 3 generations, were identified from the medical records, contacted for their compliance in our experimental studies, and as many of both the infected and non-infected members of these families bled. Their PBMC's were then utilised for various functional and genetic analyses. The following assays were then performed:

[1] Both radiometric and CFU method of assessment were used to evaluate mycobacterial (BCG) growth in monocyte-derived macrophages, as one potential parameter of cellular dysfunction which may be associated with enhanced susceptibility to infection.

[2] Titres of TNF–α production in the supernatants of monocyte-derived macrophage cultures from family members and controls were also evaluated by specific ELISA to look for differential cytokine secretion as another possible marker of cellular dysfunction and susceptibility. Cell supernatants were sampled following stimulation of cells with either recombinant IFN–γ and/or LPS.

[3] An attempt was made to correlate differences in mycobacterial growth with genetic markers segregating to the infected individuals. This was performed by linkage analysis using a panel of markers in the region of human chromosome 2q33-q37 known to be conserved with the Lsh/Ity/Bcg-containing region of murine chromosome 1. Recently moreover a candidate murine BCG resistance gene, designated "Nramp" (natural resistance–associated macrophage protein) has been cloned [Vidal *et al,* 1993] and studies of its human homologue are currently in progress. EBV transformed B-cell lines were then generated from family members and healthy controls as a self renewing source of DNA to identify resistance and susceptibility genes to tuberculosis, comparable to the Bcg gene[s] of mice. These latter genetic studies were performed in Prof. Jenny Blackwells laboratory in the Dept. of Medicine, Addenbrookes, Cambridge, as part of an ongoing collaborative study. Only the macrophage functional assay data however will be outlined in this chapter as the genetic analysis of the family material is still in progress.

7.2 Materials & Methods

7.2.1 TNF-α stimulation assays

PBMC's from each member of the TB families and healthy controls were plated out at 5 x 10^5cells/well in 200μl aliquots of RPMI-1640 + 10% autologous serum in 96-well flat-bottomed microtitre plates as described in detail in section 2.2.2. The cells were then primed with 1000U/ml of IFN-γ for 18hrs and then pulsed with 1μg/ml of LPS for 3hrs. Aliquots of 150μl/well of supernatant were then transferred into Eppendorf tubes and centrifuged at 1200g for 10 minutes, and stored at -20°C until required for TNF-α assessment by ELISA. The following controls for each family member were then included in the experimental design, i.e. IFN-γ alone, LPS alone, IFN-γ plus LPS, cells and medium alone.

7.2.2 Intracellular BCG growth/inhibition assays

PBMC's from different family members of patients afflicted with tuberculosis were isolated by Ficoll-paque separation as previously described (section 2.2.2). The cells were plated out at in antibiotic-free RPMI-1640 + 10% autologous human serum in 96-well round bottomed tissue culture plates at a concentration of 2.5 x10^6/ml in 200μl aliquots. Non–adherent cells were removed after 4 hours by repeat washing and the %–adherence determined for each individual's cultures (details of method given in section 2.2.3). The monocyte monolayers were then incubated for 4 days/37°C/5% CO_2 to allow for differentiation into mature macrophages. Four-day old macrophage cultures were then infected with pre-opsonised BCG for 4 hrs/37° C/5% CO_2 at an infection ratio of 5 BCG/cell as given in section 2.2.4.

Non-phagocytosed bacteria were removed by repeat washing and the infected monolayers resuspended in 200μl of fresh media. At zero-time, i.e. 4 hrs post-infection and at 4 days post-infection, sets of triplicate wells were harvested by lysing the cells with 0.2% saponin [wt/vol., final concentration] for 0.5 hr/37° C/5%CO_2. Intracellular BCG growth and viability was then determined for each sample by [^{3}H] uridine uptake [section 2.2.7] and CFU determination respectively, as described in section 2.2.5.

7.3 Results

7.3.1 Stimulation of TNF–α from human PBMC

PBMC's from different members of TB families were stimulated with either IFN–γ or LPS, or a combination of IFN–γ & LPS. TNF–α titres in the culture supernatants were determined by TNF–α specific ELISA. TNF assays were performed in the labs of Prof. Levin, St. Mary's Hospital, London. The results suggested that almost all the macrophage donors tested could produce detectable TNF–α in response to IFN–γ, LPS or IFN–γ & LPS in combination (Figure 7.1a). There was however no obvious pattern of correlation found between the variable patterns of TNF–α production obtained and the active disease status of the infected family individuals, suggesting marked individual heterogeneity in TNF–α production among donor macrophage populations in response to such stimulation (Figure:7.1b).

7.3.2 Intracellular BCG growth within cultured monocyte-derived macrophages from multicase TB afflicted families and healthy members

Intracellular BCG growth within 4-day old human macrophages from multicase families members where more than one family member had tuberculosis, was studied, using both [^{3}H] uridine incorporation into metabolically active bacteria over a 72hr period and CFU estimation after 14 days of culture by the microcolony counting method, as described in section 2.2.5. The intracellular infecting number of BCG was then determined at time zero [0] and the subsequent growth/survival of BCG assessed at day 4 post-infection.

Seven separate families, with different ethnic origins, consisting of 55 donor members were studied. The results illustrated as a measure of percentage change in bacteria growth in Table 7.1, indicate that individuals from 4 out of 5 families were able to control the growth of intracellular mycobacteria over the time period of the assay, i.e. 4 days. The reduction in the metabolic activity of bacteria as witnessed by [^{3}H]

Figure 7.1a
TNF-alpha production from human PBMC from memebers of multicase TB families

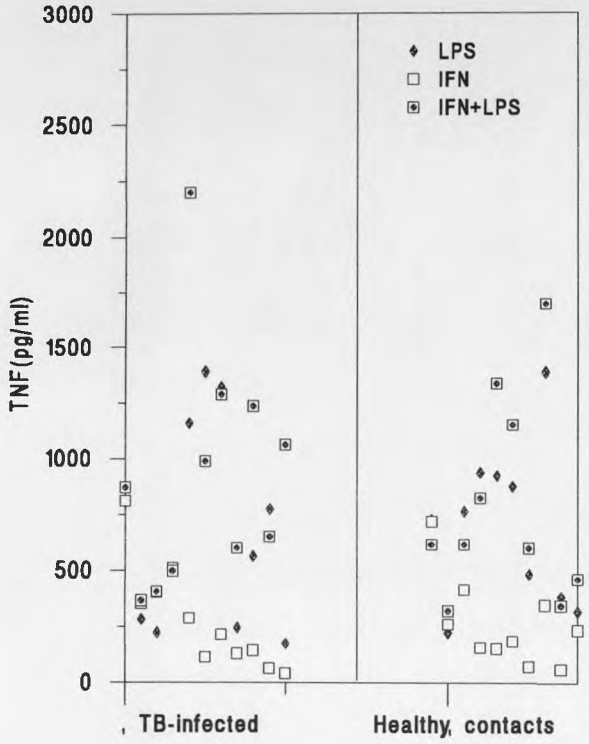
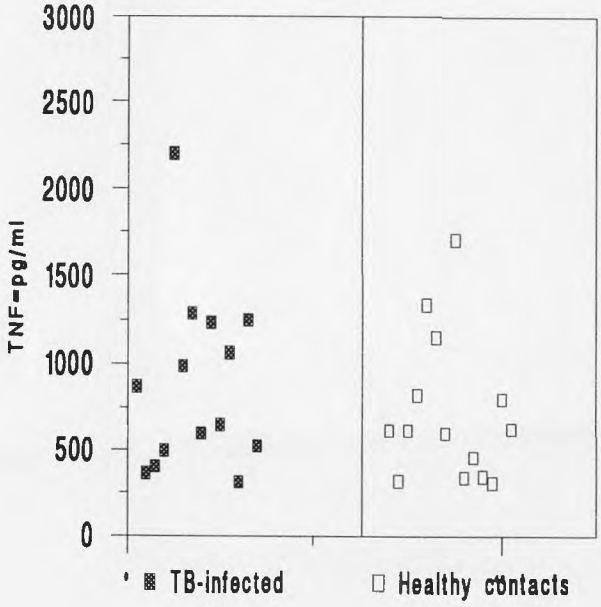


Figure 7.1b
TNF-alpha production by human PBMC on pre-activation with IFN-gamma + LPS



Figures 7.1(a & b)

These figures illustrate the pooled data of TNF-α release from PBMC from TB-infected and un-infected healthy contacts from 6 different family members following activating regimens as mentioned in the text to induce TNF–α stimulation (Figure 7.1a). Figure 7.1b however illustrates TNF-α release from PBMC following activation with 1000U/ml of IFN-γ overnight (16hrs) and 1 μg/ml of LPS (3 hrs) in both TB-infected and un-infected healthy contacts described in two groups. Each data point in both figures a & b represent TNF–α production (pg/ml/10^5 cells) obtained from the mean value of triplicate samples.

uridine incorporation was confirmed with actual reduction in growth/viability of the mycobacteria determined by CFU estimation (Table 7.1). On an average the percentage growth reduction in macrophages from TB-infected individuals was -37% in cpm and -50% in CFU, whereas in case of related un-infected healthy contacts it as -37% in cpm and -51% in CFU. The macrophage donors from family no 6 although exhibited -29% and -56% reduction in cpm but oppositely +50% and +45% growth enhancement in CFU respectively (Table 7.1). However on a whole the macrophages from TB-infected individuals were not compromised in checking the growth of intracellular mycobacteria as compared to healthy controls. It is however of interest to note that all these families were of Asian ethnic origins.

On the contrary the results from a Caucasian family (Family No: 1) as illustrated in Table 7.1 and Figure 7.2 showed that 7 out of 8 of TB-infected individuals exhibited high rates of intracellular BCG growth (+380 to +830%) within their macrophages. Of the healthy family individuals 4 out of 6 showed inhibition of intracellular BCG growth (-2 to -9%). One exhibited a high rate of intracellular growth (+500%) comparable to that shown by infected family members and the last healthy subject showed an intermediate level (+155%) of intracellular BCG growth (Table 7.1). It is worth mentioning that these results were not confirmed by CFU estimate.

7.3.3 Lack of correlation between TNF-α release and intracellular BCG growth pattern

TNF-α production following IFN–γ & LPS-activation of PBMC from both TB-infected individuals and un-infected healthy contacts was compared to corresponding percentage change in intracellular BCG growth as determined by CFU estimate. The results as illustrated in Figure 7.3 fail to show any correlation between TNF-α release and intracellular BCG growth.

Table 7.1
Percentage change[a] in the intracellular BCG growth within human monocyte-derived macrophages from TB-infected and un-infected multicase TB family members and healthy contacts.

Families	Macrophage donors	cpm[b]	CFU[c]
1 TB-infected	1	+500	ND[d]
	2	+300	ND
	3	+830	ND
	4	+800	ND
	5	-2	ND
	6	+380	ND
	7	+840	ND
	8	+500	ND
Healthy contacts[e]	9	0	ND
	10	-9	ND
	11	+500	ND
	12	-2	ND
	13	-2	ND
	14	+155	ND
2 TB-infected	1	-41	-14
	2	-16	-40*
	3	-44	-71*
	4	-41	-58*
Healthy contacts	5	-25	-59*
	6	-9	-58*
3 TB-infected	1	-39	-68*
	2	-14	-44*
	3	+10	-39*
	4	-37	-36*
	5	-8	-48*
Healthy contacts	6	-27	-39*
	7	-10	-63*
	8	-17	-37*
	9	-42	-39*
4 TB-infected	1	-41	-87*
	2	-60	-83*
Healthy contacts	3	+15	-84*
	4	-38	-88*
	5	-44	-87*
	6	-53	-87*
	7	-46	-86*
5 TB-infected	1	-33	-28
	2	-11	-46*
	3	-89	-50*
Healthy contacts	4	-54	-37*
	5	-36	-25
	6	-57	-47*
	7	-19	-22
6 TB-infected	1	-29	+50**
	2	-56	+45**
Healthy contacts	3	-33	-55*
	4	-16	-61*
	5	-2	-67*
	6	-47	-4
7 TB-infected	1	-24	-34*
Healthy contacts	2	-44	-35*
	3	-54	+35**
	4	-50	-11
	5	-57	-38*
	6	-33	-48*

[a]Percentage change in bacterial growth = [1- cpm or CFU at day 4 / cpm or CFU at zerotime] x 100.
+ represents growth enhancement, - growth reduction.
[b]Counts per minute (cpm) obtained by [^{3}H] uridine incorporation.
[c]Colony forming units (CFU) obtained by Microcolony counting method.
[d]ND = not determined.
[e]Healthy un-infected contacts were taken as controls from the same family.
* Statistically significant growth reduction ($p < 0.5$)
** Statistically significant growth enhancement ($p < 0.5$).

This table represents percentage change in bacterial growth as determined by radiolabelling and CFU estimate. Four-day old human monocyte-derived macrophages were obtained from different members of the TB-afflicted families, and infected with BCG at an infection ratio of 5 BCG/macrophage. Extracellular bacteria were removed, and intracellular bacteria were determined 4 hours post-infection as zerotime (0), both by [^{3}H] uridine incorporation and CFU estimate. At day 4 post-infection, the growth/survival of bacteria was assessed, and percentage change in bacterial growth was calculated as given in the footnote. Statistics (Students *t*-test) were performed for CFU values only, which are indicative of viability of bacteria.

Figure 7.2
Intracellular BCG growth in a Caucasian family (No: 1).

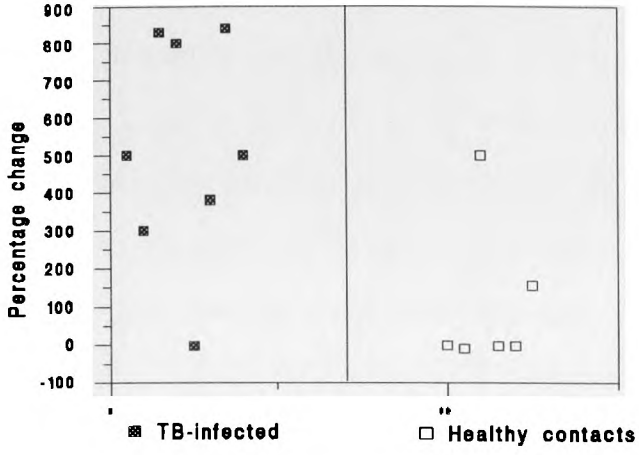


Figure 7.3
Correlation between TNF-alpha production and percentage growth inhibition of intracellular BCG.

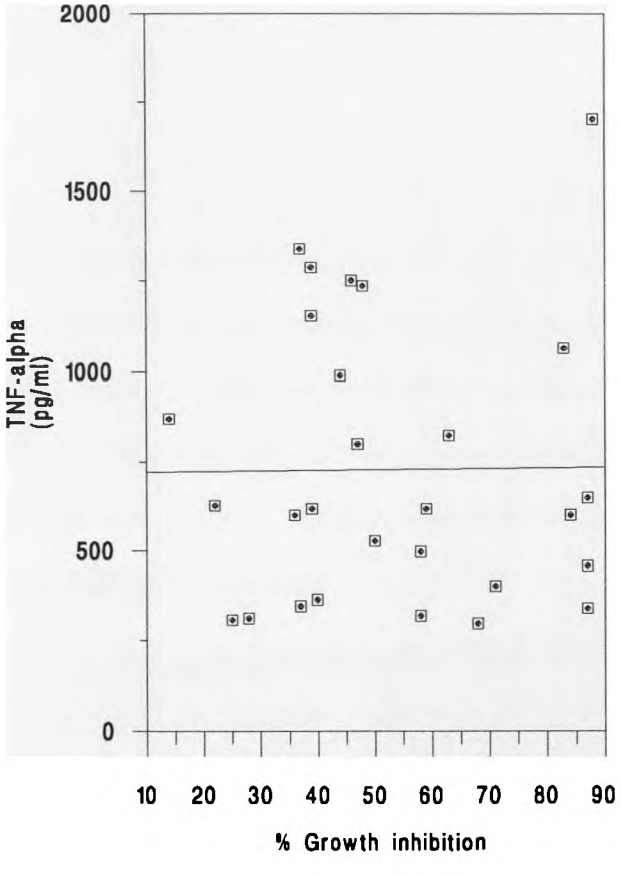


Figure 7.2

This figure illustrates intracellular BCG growth in 4-day old human monocyte-derived macrophages from a Caucasian family (Family No: 1) desribed into two groups of individuals, i.e. TB-infected and un-infected healthy contacts. Macrophage monolayers were infected with BCG at infection ratio of 5 BCG/cell. Extracellular BCG were removed and intracellular infecting bacteria were determined by [^{3}H] uridine incorporation at 4 hours post-infection as zerotime (0). Intracellular BCG growth was determined by harvesting the infected cell cultures at day 3 post-infection. Each macrophage donor was tested in triplicate. Percentage change in bacterial growth was then calculated by the following equation = [1- cpm (day 4) /cpm (zerotime)] x 100

Figure 7.3

This figure represents correlation between TNF release (x-axis) by human PBMC from TB-infected and un-infected healthy contacts and percentage change in bacterial growth as determined by CFU estimation (y-axis). TNF values (pg/ml/10^5 cells) were obtained post-stimulation with 1000U/ml of IFN-γ for 16 hrs and 1μ g/ml of LPS for 3 hrs. Each data point of TNF-α release represents mean of triplicate values as determined by ELISA assay. The percentage change in intracellular BCG growth was calculated by the following equation = [1- CFU (day 4) /CFU (zerotime)] x 100.

7.4 Discussion

To investigate the theory that individuals from identified multicase TB families have an innate macrophage-based susceptibility to mycobacterial infections, members of 10 such families were studied for cellular dysfunctions. A Caucasian family of 14 from Cumbria, with 8 members in three generations who had developed tuberculosis were identified and sampled for analysis. Peripheral blood monocyte-derived macrophages from 7 out of 8 diseased subjects, were shown to permit high rates of intracellular BCG growth, while 5 out of 6 asymptomatic family members exhibited relatively low rates of intracellular BCG growth, comparable to those within unrelated healthy control subjects. Forty one members of 9 other multicase Asian families, identified in the West Midlands, were also similarly tested, but showed no such positive bacterial growth correlation's with infection status. The differences observed between the Caucasian and Asian family findings may be attributable to the documented racial differences in susceptibility and background prevalence of the infection in the two ethnic groupings. Multicase Caucasians families, with the exception of those associated with known HIV+ involvement, are rare, and coupled with the low background incidence of the disease in the UK, may represent a genuine genetic inheritance of a putative TB susceptibility gene[s]. Whereas, within Asians, the higher incidence of such multicase families in this ethnic grouping is thought to be more attributable to the higher environmental exposure to the disease of such individuals following immigration of family members from the Indian subcontinent, rather than to any inherent genetic effect.

The mechanism of host resistance to another intracellular pathogen, *Brucella abortus* in cattle has also been shown to be genetically controlled [Price *et al,* 1990]. Naturally Brucella resistant or susceptible individuals have been identified within outbred herds, and the phenotype is at least partially expressed at the level of host macrophages. This functional defect was determined in a similar fashion to that described in our human studies, in that monocyte-derived macrophage cultures were prepared from both resistant and susceptible cattle and their differential bacterial loads determined following *in vitro* challenge with a standard infection of *B. abortus*. The results of the Brucella study bear a strong resemblance to those found

with the Cumbria family in this study, in that significantly higher bacterial loads were observed in susceptible individuals as opposed to resistant cattle. The cattle findings then parallel the situation described in mice and humans, the system differs in cattle however, because a multigenic control of resistance to infection has been postulated [Price *et al,* 1990]. However a portion of the bovine chromosome 8 has been identified that is syntenic with the conserved sequences found on mouse chromosome 1 and human chromosome 2q. The search for the Bcg homologue may then be carried over to live stock populations, with potentially important consequences for the agricultural industry in terms of breeding programmes for selection of resistance to a whole range of intracellular pathogens.

Assessment of TNF production by PBMC populations isolated from any of the infected and non-infected family members and healthy controls, showed no consistent pattern or correlations with either infectious status, or in the case of the Caucasian families, with higher intracellular bacterial growth rates. This is at odds with previous data cited from murine studies where macrophages derived from Bcg-resistant mouse strains produced more TNF–α on stimulation, as compared to Bcg-sensitive strains [Orme *et al,* 1993a]. Furthermore, human studies undertaken by Prof. Michael Levin's group at the Dept. of Paediatrics, St Mary's Hospital , London, have identified a group of Maltese children with familial disseminated atypical mycobacterial infections. These children, unlike the Cumbria or Asian family members examined in this study, do have a demonstrable defect in their monocyte/macrophage function, such that their monocytes and macrophages are unable to produce adequate amounts of TNF in response to a variety of stimuli, including endotoxin. Their cells are also unable to up-regulate TNF-α in response to gamma interferon [Levin personal communication]. The defect however is also associated with abnormal T cell proliferative responses to mycobacterial antigens, which was not shown to occur in our TB multicase family patients [Kumararatne personal communication].

Recent studies moreover have reported marked genetic polymorphism of the TNF genes in humans which may be important in view of the putative link between these genes to MHC–associated human diseases with an inflammatory component such as TB [Wilson *et al,* 1993]. An association between TNF genes and

disease may then reflect an altered or disrupted control of TNF gene expression. Indeed in some autoimmune disease, alterations in TNF gene expression have been noted [Wilson *et al,* 1993].

In retrospect therefore it may have been more informative, i.e. accurate, to have analysed TNF secretion within purified macrophage cultures from each of the family members, rather than using stimulated PBMC, thereby avoiding the influence of any concomitant T cell dysfunction on the results. Moreover, it would also have been interesting to have looked at a broad panel of monokines in response to IFN–γ and/or LPS stimulation, to identify in more detail possible differences in cytokine profiles between cells from recurrent infected and non-infected siblings, rather than to have looked only at TNF–α secretion. Moreover, cytokine responses to a live infection or to LAMs from virulent and avirulent *M. tuberculosis* strains may also have been more appropriate, given that cellular responses to LPS are also known to be genetically regulated in mice [Fultz & Vogel, 1989]. The use of live infections or LAM stimulation, especially in analysis of the Cumbria family, to look for correlations with the observed bacterial growth inhibition in infected members, may then have proven more informative. Mycobacterial LAMs for example are known to exhibit immuno-modulatory effects, favouring mycobacterial survival *in vivo*. These include; scavenging toxic oxygen radicals, inhibition of macrophage activation and interference with antigen processing and in consequence, suppressing T cell activation. Recent studies in our laboratory have also shown that LAMs from virulent strains of *M. tuberculosis* are less potent at inducing protective cytokine production, especially TNF–α from human macrophages compared to those from virulent mycobacteria, indicating that the structure of LAM from a given mycobacterial strain may influence its ability to elicit or avoid cytokine-mediated host resistance.

The cytokine networks which operate between T cells and monocyte/macrophages following activation or infection and are known to be complex, involving both positive and negative signalling. Moreover there appears to be major differences in the cytokine regulation of anti-bacterial immunity expressed by murine and human macrophages [Reviewed in Moreno & Rees, 1993]. Murine anti-mycobacterial immunity is thought to be much more efficient than in man. Murine macrophages moreover have been reported to be

readily activated *in vitro* by cytokines to inhibit bacterial metabolism and growth [Moreno & Rees, 1993]. In contrast, activation of human macrophages by a variety of recombinant lymphokine regimes have been found to produce only modest inhibition of mycobacterial growth at best, and we have shown that the degree of production of nitric oxide by human macrophages is much lower than in mice, and varies from donor to donor [Chapter 6]. It is of interest in this context that the murine N-ramp gene is thought to code for the expression of a macrophage membrane nitrate transporter protein [Vidal *et al*, 1993]. Activation of human cells by IFN–γ moreover has been observed in this and other studies to result in higher bacterial growth than in non–activated cells [reviewed in Kaufmann, 1993]. This has been proposed to reflect the interaction which may occur in human cells between IFN–γ, and IL-12 as positive activation signals, and with IL-4, TGF-β or IL-10 as known, down regulators, or negative signals on macrophage activation [Moreno & Rees, 1993].

The interplay between the T cell influences upon macrophage responses to infection with mycobacteria and inherent genetic effects regulating macrophage function and activation are therefore probably very complex and subtle, and require further dissection and analysis. A possible better candidate as a cytokine marker correlating with macrophage-based genetic susceptibility or resistance to intracellular infections such as TB is interlukin-12. In recent murine studies, macrophages from Bcg/Ity/Lsh resistant mice infected with appropriate intracellular pathogens, have been shown to secrete IL-12, that induces natural killer (NK) cells to produce IFN–γ, whereas macrophages from susceptible strains lack this capacity [Ramarathinam *et al*, 1993a]. TNF moreover has been shown to enhance this effect [Ramarathinam *et al*, 1993b]. Interleukin-12 is a heterodimeric cytokine produced by monocyte-macrophages, B cells and other accessory cells, in response to bacterial products or parasites [Trinchieri, 1984, Trinchieri *et al*, 1993]. It has pleotropic effects on NK cells and T cells, including induction of transcription and secretion of cytokines; enhancement of cytokine activity and induction of proliferation of T and NK cells [Scott, 1993]. Those pathogens associated with TH1 cell responses or development furthermore, have been shown to be the same as those inducing IL-12 production in murine and human macrophages including; *Staphylococcus aureus* & Toxoplasma [Scott, 1993].

Current hypothesis then is that on infection with intracellular pathogens, monocyte-macrophages respond by producing TNF, IL-1 and IL-12 In turn, IL-12, in co-operation with TNF & IL-1, is thought to act on NK and TH-1 cells to induce IFN–γ production; and to induce differentiation of TH0 precursor cells to TH-1 cells in primary response. IFN–γ in turn acts back on macrophages to up regulate TNF–α receptor expression, thereby allowing up-regulation of macrophage activation by TNF, either autocrine or T-cell derived. IFN–γ is also thought to have a positive feed-back effect on macrophages by enhancing production of IL-12. Conversely, TH-2 associated cytokines IL-10 & IL-4 are known to have a negative feedback effect by acting as powerful inhibitors of IL-12 production and upon the activation effects of IFN–γ on macrophages. The effect of IFN–γ in enhancing IL-12 might also be in part, be mediated by its ability to block endogenous IL-10 production by macrophages. It is likely then that IL-12 will prove to play a more central role in linking innate natural resistance, mediated by phagocytic cells & NK cells, and adaptive immunity, mediated by Th cells, cytotoxic T cell and possibly B cells in mycobacterial infections, and in directing the resulting CD4+ T helper-cell development pathway. Any further studies on possible innate differential cytokine signal production by monocyte/macrophages, from genetically susceptible and resistant individuals to tuberculosis, will then have to evaluate IL-12. The *in vitro* macrophage culture system utilised in this study however still offers a good model system to undertake such an investigation.

The role of TNF in innate-resistance to infection with *M. tuberculosis* however is still worthy of further investigation as together with the previously cited murine studies. Human infections, are characterised by cachexia, a prolonged acute phase response (high ESR) & T cell recruitment to granuloma, all features attributable to TNF production. Cadranel *et al,* [1993] moreover reported that peripheral blood monocytes from patients expressed greater numbers of TNF receptors than controls, and that a greater proportion of them complexed with TNF. This provides evidence for a generalised macrophage activation in TB, and of increased TNF synthesis. These finding moreover complement those previously reported by Ogawa *et al,* [1991] who showed that that patients peripheral blood monocytes produce more TNF than healthy controls. They also invite comparison with murine studies of Kindler *et al,* [1989] in the studies of TNF levels in mice infected with BCG. A recent abstract by Friedland *et al,* [1993], however has described the results of the assessment of TNF–α responses in 41 TB patients in Zambia, following initial diagnosis and after 2

months antimycobacterial therapy. It was revealed that leukocytes from patients who died, which were stimulated *ex vivo* with LPS in whole blood assays, released less TNF than patients that recovered, and that cytokine secretion patterns in surviving patients normalised with therapy. The results were considered to represent that a critical impairment of host immune defences occurred in those individuals who were unable to respond to chemotherapy and suggested a protective role for TNF in immunity to TB infections.

Further studies are then needed to define the physiological and molecular basis of innate resistance and susceptibility to TB, thus paving the way for better targeted strategies of vaccination and treatment of genetically susceptible subjects. Evidence for success of the latter approach has recently been described by [Holland *et al,* 1993], where IFN–γ has recently been reported to be of benefit for the treatment of atypical mycobacterial patients, not associated with HIV infection. Such individuals are also thought to have a genetic susceptibility to infection with mycobacteria, which has been associated with a demonstrable inability of their PBMC to produce sufficient IFN-γ on challenge *in vitro*, as compared to cells from normal healthy controls [Holland *et al,* 1993]. The therapeutic effect of IFN–γ treatment however was not thought to act directly upon macrophages, to correct a cytokine deficiency, although such patients were also found to produce poor TNF–α responses on challenge of their PBMC's in a whole blood assay [Holland *et al,* 1993]. Understanding further the cellular dysfunctions in such patients may then help to identify the protective mechanisms involved in resistance to infection and suggest more rational approaches to effective therapeutic strategies.

CHAPTER 8

General Discussion & Conclusions

The main aims and objectives of this thesis were to look at and evaluate the effector functions which may be involved in human mycobacterial immunity. Since most of the methods and results of such studies relied critically upon the accuracy, reliability and reproducibility of the methods of determination of the growth and viability of mycobacteria, extensive efforts were initially undertaken to assess the currently available methods. These involved either, colony-counting or radio-labelling of organisms released from disrupted cells. We then compared the different available methods of assessing the growth of BCG/MAI in macrophage-free and macrophage-treated systems by radiolabelling, with [^{3}H] uracil, [^{3}H] uridine, and [^{3}H] glycerol, and CFU counting either by agar plating or by a new technique of microcolony counting in broth. All the methods examined were found to detect BCG/MAI growth/viability to varying degrees and the sensitivity range of each were determined.

What quickly became apparent from these assessments was the numerous technical difficulties involved in accurately determining intracellular bacterial killing within human cells. What was also surprising was the number of reports in the literature, which had erroneously claimed that murine or human cells could kill various mycobacterial species, either following cytokine activation or by specific mediators, such as ROI or RNI, based purely on radioisotope incorporation studies. Whereas in fact, in our opinion, these could be at best, be interpreted as bacteriostasis. Radioisotope labelling, while being a very rapid and easy to assess method of determining bacterial growth, was not found to be capable of accurately measuring bacterial viability or survival. Therefore, to be reliable, any results of radioisotope incorporation always needed to be supported and confirmed by CFU determination. The development of the microcolony counting assay was then of great assistance and importance to our ability to more rapidly and economically assess large numbers of replicate samples from cell lysates for bacterial load and viability. Together with [^{3}H] uridine radiolabelling, this combination of methods proved to be the best composite technique available to determine intracellular mycobacterial growth and viability following uptake of the organism into human monocyte-derived macrophages.

When these studies were first initiated nearly four years ago, the current dogma was that in murine and human hosts, mycobacteria were killed by reactive oxygen intermediates produced by activated macrophages

[Mackaness, 1969]. This hypothesis however was questionable even then, and we attempted to re-examine it in a novel way, by undertaking a comparative assessment of mycobacterial survival in neutrophils and macrophages from Chronic Granulomatous Disease (CGD) patients. Phagocytes from CGD patients, which have a genetic enzyme deficiency and thus are unable to generate O_2 radicals were however shown to have similar intracellular BCG growth rates as was obtained in cells from normal healthy controls. This strongly suggested that ROI do not in fact play a critical role in the regulation of mycobacterial infections in human macrophages.

In subsequent experiments, human monocyte-derived macrophages were pre-activated with different cytokine regimens to look at their effect on mycobacterial survival. These included, IFN-γ, and/or LPS, and CPS (Con A-stimulated PBMC supernatant).

However, after the failure of all such treatments to induce mycobactericidal activity in human macrophage cultures, the studies were continued but with an alternative strategy of using cytokine inhibitors. TNF–α release in BCG-infected human monocyte-derived macrophages was then effectively shown to be blocked by a number of different agents including Thalidomide, Oxpentifyline, Dexamethasone and an anti-TNF-α polyclonal antibody, as determined by ELISA. There was however no significant enhancement of intracellular BCG/MAI growth over a 7-day incubation in human macrophages in the presence of any of the four agents used. The result then suggests that the action of TNF–α alone is also unlikely to be an important effector mechanism in antimycobacterial immunity within human cells. This finding was further supported by functional studies on members of seven multi-case TB families, where there was found to be no discernible correlation between TNF-α release, following stimulation with IFN-γ and/or LPS, and disease status within the quasi genetically susceptible individual family members. Furthermore, preliminary results from these studies suggested that in 6 out of 7 of the multicase families, there was also no difference in the control of intracellular mycobacterial growth, at a macrophage level, between infected and non-infected individuals and healthy controls.

Other anti-mycobacterial effector mechanisms, previously identified in mice, were then also investigated in this thesis, using our *in vitro* human cell assay system. These included the possible involvement of reactive

nitrogen intermediates (RNI) in the intracellular killing of mycobacteria within murine and human macrophages. However in our assays, all attempts and treatment protocols examined, which have been previously shown to be effective with murine cells, failed to induce NO in human macrophages. This was confirmed by the lack of effect of the NO-synthase inhibitor, LNMMA, to affect the intracellular growth of BCG in infected human macrophages, as compared to non-treated but infected control cells. Furthermore, within this series of experiments the novel approach of using strains of *M. smegmatis* with varying capacities to produce the enzyme Catalase, was utilised, to help determine an effector role for this enzyme on the NO-pathway, in regulating mycobacterial infections in murine and human cells. The murine cell line, J774 was then readily shown to produce high levels of detectable nitrate synthesis, yet failed to control the intracellular growth of any of the *M. smegmatis* strains examined. No nitrate release moreover was detectable from human phagocytes, yet interestingly, they were shown to initially retard the growth *M. smegmatis* over the first 24 hrs post-infection. At later time points however, they also failed to check the growth of the mycobacteria.

After a series of failed attempts to dissect the macrophage-based effector function, an alternative strategy was investigated, i.e. to examine the role of cytolytic T cells in immunity to mycobacterial infections. Human CD4+, mycobacteria-specific, cytolytic T-cell clones were then used to lyse BCG-infected macrophages, and the effect on the subsequent growth and viability of the organisms was examined. The survival of released bacteria following cell lysis was assessed by both [^{3}H] uridine labelling and colony forming unit (CFU) estimation. The results however indicate that even when effective antigen-specific or lectin-mediated cytolysis of the infected macrophages was achieved, there was no evidence for a direct mycobactericidal effect on the intracellular bacteria. This remained the case even if the period of co-culture of T cells and macrophages was extended up to 48 hours. Pretreatment of the macrophages with interferon-gamma was not able to act together with T cell-mediated lysis to produce inhibition of mycobacterial growth. Moreover re-phagocytosis of BCG by fresh monocytic monolayers also did not reduce the growth/viability of the organisms.

A worrying aspect however of all the negative findings reported in this study, is the nagging doubt of whether any *in vitro* model system of determining the immune mechanisms operating in human cells, is to simplistic to recreate the complex and delicate balance of factors and cells that are undoubtedly interacting in the *in vivo* situation. Therefore, one really now needs to ask the question, are human macrophages really unresponsive to the *in vitro* action of recombinant cytokines because: (i) they do not respond to these signals, or (ii) that they require a subtle balance of signals, or (iii) that they require further co-stimulatory signals not available to them in the *in vitro* system, or (v) that the apparent lack of effect is purely an artefact of a deficient *in vitro* culture system, or (iv) that their lack of mycobactericidal activity is an accurate portrayal of this dysfunction *in vivo* and that human phagocytes do not, or cannot kill mycobacteria!

Furthermore, does any of this negative data actually help us identify what causes the inhibition or control of mycobacterial growth in human infections ? From population studies we know that only 20% of people who become infected with TB go on to develop clinical disease [Snider, 1989], suggesting that the innate and specific immune mechanisms which have been proposed to operate in most individuals, are indeed effective in controlling this infection in the majority of the population. Moreover, the recent upsurge in TB in Africa, associated with the AIDS epidemic, also provides strong evidence for the importance of T cell-mediated immunity in controlling tuberculosis in humans [Kumararatne *et al,* 1992]. Data from this thesis moreover, at least with the Cumbria multi-case TB family, would also suggest that macrophage-based resistance to infection, probably does play a part in a natural innate immunity to TB. Assuming then, that these different pieces of evidence give an accurate indication of the effective antimycobacterial mechanisms operating in man, then cytokine-activated macrophages harbouring intracellular organisms may still be validly proposed to prevent microbial replication. These could be hypothesised to achieve this objective in at least four ways: (i), by creating an intracellular environment which is unsuitable for reproduction. This could for instance be due to low pH or lack of critical nutrients, such as iron, in the phagosome. (ii), by a killing reaction active against only dividing bacteria. This would have the net effect of producing zero population growth due to the combined events of equal intracellular growth and killing. It would also put a selective pressure on the organism to develop dormant non-dividing forms to survive until conditions are more conducive to replication. (iii), the activated macrophage may produce microbiostatic effector molecules at a slow and steady pace, such as NO, thereby maintaining the intracellular organisms in a non-replicating state. (iv),

that mycobacteria are killed by repeated cycles of phagocytosis within activated monocyte/macrophages, following repeated cell mediated lysis, either by specific CD8+ or CD4+ T cells.

Conversely, a number of virulence factors have been proposed to contribute to the intracellular survival of mycobacteria within phagocytes. These include: (i) the outer glycolipid layer of the mycobacterial cell wall conferring resistance to active oxygen radicals and other microbicidal mechanisms [Brennan, 1989]. (ii) lipoarabinomannan cell wall components of virulent mycobacteria binding to specific cell surface receptors, e.g. mannose, or fibronectin receptors, thereby allowing silent entry into a cell, i.e. not activating the normal proinflammatory cytokines generated in response to avirulent strains [Chatterjee *et al,* 1992] (iii) Superoxide dismutase, inactivates reactive oxygen intermediates. (iv) Prevention of phagosome-lysosome fusion following phagocytosis of mycobacteria. (v) The ability to develop dormant forms which have low metabolic activity and can persist *in vivo*, intracellularly for many years [Toman, 1981]. (vi) The ability of virulent strains to escape from the phagolysosome into the cytosol (vii) Inhibition of T cell responses by mycobacterial cell wall components, e.g. lipoarabinomannan, by either an antigen presentation-mediated inhibitory effect on lymphocyte activation, or by the stimulation (?) of suppresser T-cell function [Ivanyi, 1986, Ellner & Wallis, 1989). (viii) That mycobacteria may induce apoptosis within infected macrophages to aid the spread of the organism from one macrophage to another without re-entering the hostile extracellular milieu, such as been recently proposed for virulent Shigella strains [Zychlinsky *et al,* 1992]. Lastly, it has been proposed that the expression of a recA gene product may aid the intracellular survival of *M. tuberculosis* [Davis *et al,* 1992] as such genes are known to encode for DNA repair mechanisms and other SOS functions, permitting bacterial survival within an otherwise hostile environment.

In conclusion, therefore, although it is generally accepted that resistance to tuberculosis is mediated by specific T-lymphocytes and expressed by activated macrophages, the signals and mechanisms involved in determining the outcome of macrophage-mycobacteria interactions in man are still unresolved and await critical evaluation.

As a suitable end to this thesis, I would then like to highlight and review, various aspects of interest in current TB research, which I would consider to be worthy of further investigation in any subsequent project in this area. These include:

1) The role of CD8+ T-cells in host immunity to infection with mycobacteria, following the finding that "Knock-out " mice which are unable to generate CD8+ T cells due to a targeted disruption of the β_2-microglobulin gene, when given a normally sublethal dose of *M. tuberculosis* succumb to a progressive and disseminated disease [Flynn *et al,* 1992]. Not only does this model represent the first of the "new wave" of models based upon specific gene targeting, but it also revives interest in the possible role of CD8+ immune T cells in TB infections. Such a protective role is also suggested by the findings of Lowrie, [1993] who showed that in mice which had been infused with a macrophage cell line transfected with the gene encoding expression of *M. leprae* hsp 60, that the effector T cell population triggered to counter a lethal infection with *M. tuberculosis* were CD8+ cells [Lowrie, 1993].

2) The role of polyamines in controlling intracellular mycobacterial infections in human cells following recent evidence for their involvement in producing such an effect in cells isolated from guinea-pigs made resistant by multiple vaccination [Andrews, 1993].

3) The use of human probes to the newly identified Nramp gene in mice, as a human homologue of the murine Bcg gene. The Nramp gene in mice is thought to encode for a nitrite transporter protein [Vidal *et al,* 1993], which would tie in very well with the well documented role for nitric oxide in murine phagocytes. It would therefore be of great interest to identify what the human homologue codes for, and which may re-open the debate on the role of NO in human cell systems.

4) The finding that the lipoarabinomanans (LAM) from the cell walls of virulent and avirulent strains of mycobacteria differ in the percentage of mannose capping, such that virulence equates with greater mannose [Chatterjee personal communication]. Coupled with the further demonstration that these LAM's on their own, are capable of differentially stimulating TNF and other pro-inflammatory markers *in vitro* in mice [Roach *et al,* 1993] and humans [Lammas personal communication] suggests that these may then act

to aid the silent entry of virulent strains into cells via mannose receptors. Hence, by not activating the cell to the same degree as avirulent strains, may give the edge to their survival within the phagocyte environment. Recently it has been found that macrophage phagocytosis of virulent but not attenuated strains of *M. tuberculosis* is mediated by mannose receptors in addition to complement receptors [Schlesinger, 1993].

5) The reported observation that virulent strains of *M tuberculosis*, are able to gain entry to, and multiply within the cytosol of infected cells [King *et al,* 1993]. This was thought to be achieved through production of a haemolysin-like molecule similar to that previously described in Listeria infections [Boom *et al,* 1991, 1992]. Escape from the phagolysosome would then allow the bacteria to escape from the potentially lethal effects of the lysosomal granules, and interestingly, may also be propose to result in such strains evoking a greater class-I restricted (CD8+) T cell response.

6) It is known that foci of infection, such as pulmonary granulomata of tuberculosis, are sites of rapid trafficking and turnover of monocytes and lymphoid cells, as well as lymphokine production and tissue destruction [Kaufmann, 1989]. Understanding the cellular mechanisms operating to produce protective immunity against mycobacterial infections may then depend upon an understanding of the complex dynamics of this peculiar micro environment. This in turn may occur if methods were devised to accurately reproduce such an environment *in vitro*. This could feasibly be achieved for example by the use of organ cultures, or by culturing granuloma tissue in SCID mice. Such novel avenues of approach to future research in this area deserve further consideration.

7) To follow up on findings made in this study, that mycobacteria appear to undergo a lag phase of growth within the first 24 hrs post release from human macrophages (Chapter 3). This is a potentially interesting finding, indicating that mycobacteria are under some sort of growth inhibition within the intracellular environment of the macrophage. It is also pertinent to note that it was observed during many experiments performed in this study on human cells measuring bacterial growth indices, using the microcolony assay system, to determine the CFU of BCG. Occasionally it was found that in cells from certain individuals, very few or no colonies were obtained within the highest dilution of cell lysate utilised. This suggested either that the cell homogenate was inhibitory to bacterial colony growth, and if so, why only in certain individuals, or

else that an intracellular mediator, such as the macrophage equivalent of a neutrophil defensin, was acting to inhibit bacterial growth. Such an effect would not normally be observed using standard agar plate cultures, as presumably such a mediator would diffuse away. It would be nice to repeat these findings, using cells from identified individuals, to try and isolate the mediator involved and correlate its activity to the ability of the cells to control mycobacterial infections. Finally, regarding this potential mediator, it has been observed in this laboratory that such activity is regularly observed when performing micro-colony assays on IFN-γ activated murine cells [Bartlett personal communication]. This inhibitory factor has provisionally been designated "Kumarcillin" in honour of my supervisor .

CHAPTER 9

References

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CHAPTER 10

Appendices

Appendix: 10.1 Miscellaneous materials

Donkey anti-sheep polyclonal antibody: supplied by The binding site. Ltd. The University of Birmingham Research Institute, 97, Vincent drive, Edgbaston, Birmingham, B 15 2 SQ, England.

HRP-conjugate:-kindly supplied by Mrs. Chandra Raykundalia, Department of Immunology, Medical School, Birmingham.

MAb TNF : was kindly supplied by Dr. M. J. Tarlow, Dept. of Infection, University of Birmingham, Birmingham.

Thalidomide :- Thalidomide used in this study was supplied by East Birmingham Hospital, Birmingham.

TNF-α :was kindly supplied by Dr. M. J. Tarlow, Dept. of Infection, University of Birmingham, Birmingham.

TNF- alpha antibody:- A rabbit polyclonal anti-human TNF-alpha antiserum was donated by Dr. R. Kiessling, Karolinska Institute, Sweden.

Trental :- Oxpentifylline powder was kindly supplied by Hoechst, U. K, Limited

Appendix : 10.2 ELISA buffers

All reagents were supplied by Sigma, Dorset, England. U.K.

1.Coating buffer- 0.05 M Carbonate-Bicarbonate buffer, pH = 9.6

Na_2CO_3 (Sodium carbonate, anhydrous) MW =105.99, 1.59gm
$NaHCO_3$ (Sodium hydrogen carbonate) MW =84.01, 2.93gm

The pH was to adjusted to 9.6 (Pye Model 292 pH meter) by addition of one solution into the other.

2. Diluting buffer- Phosphate-buffered saline (PBS) with 0.05% (v/v) - Tween 20 , pH= 7.4

NaCl 8.5 gm
$NaHPO_4$ 0.2gm
Na_2HPO_4 2.8gm

These were added to 1 litre of distilled water, and 0.5 ml of Tween 20 was added.

3. Washing buffer - Saline with 0.05% (v/v) - Tween 20

0.85 % saline + 0.05 % Tween 20

NaCl 45 gm
Tween 20 2.5 ml

4. Substrate buffer - 0.15M Na-Citrate buffer pH= 5.0

Citric acid = 7.3 gm
Na_2HPO_4 = 11.6 gm
These were dissolved in 1 litre of distilled water.

5. Stopping solution - 20 % Sulphuric acid

H_2SO_4 20 ml
Distilled water 80 ml

6. Substrate, OPD (Orthophenylene Diamine Dichloride)

10 mg of OPD was dissolved in 25 ml of 0.15 M citrate phosphate buffer, pH 5, 50 µl of 30 % H_2O_2 was added just before use and kept at 4°C.

Appendix: 10.3 NO_2^- production by 4-day-old human monocyte-derived macrophages

IFN-γ (1000U/ml)	LPS (10ng/ml)	*M. smegmatis* (5 bacteria/cell)	*NO_2(nM/ml) 24hrs	48hrs
-	-	+[1]	< 4	<4
-	-	+[2]	< 4	< 4
-	-	+[3]	< 4	< 4
-	-	+[4]	< 4	< 4
-	-	+[5]	< 4	< 4
-	-	-	< 4[a]	< 4[a]
+	-	-	< 4	< 4
-	+	+	< 4	< 4
+	+	-	< 4	< 4
+	+	+[1]	< 4	< 4
+	+	+[2]	< 4	< 4
+	+	+[3]	< 4	< 4
+	+	+[4]	< 4	< 4
+	+	+[5]	< 4	< 4
+	-	+[1]	< 4	< 4
+	-	+[2]	< 4	< 4
+	-	+[3]	< 4	< 4
+	-	+[4]	< 4	< 4
+	-	+[5]	< 4	< 4

[1]MC_2155
[2]BH1
[3]BH1/pBH4
[4]BH1/T35
[5]BH1/T586

*Mean of triplicate values, sd < 10% of mean (not shown)
+ Presence , -- Absence of activating agent.
[a](cells alone)

This table illustrates nitrate levels (nM/ml/5x 10^4 cells) human monocyte-derived macrophages obtained from different macrophage donors. Cells were activated with different cytokine regimens with or without *M. smegmatis* infection (5 strains). The supernatants were harvested after 24-48hrs and nitrate levels determined by chemiluminiscence assay.

Appendix : 10.4 Activation of human monocyte-derived macrophages to produce detectable NO as determined by chemiluminiscence assay.

Cells in culture (Days)	Activation regimens[c]	NO_2^- (nM/ml/5x10^4 macrophages)			
	Time post-stimulation (hrs)	1	4	18	24
1	IFN-γ	<4[a]	<4	<4	<4
	LPS	<4	<4	<4	<4
	IFN+LPS	<4	<4	<4	<4
	CPS[b]	<4	<4	<4	<4
	BCG/MAI/*M. smegmatis*[d]	<4	<4	<4	<4
4	IFN-γ	<4	<4	<4	<4
	LPS	<4	<4	<4	<4
	IFN+LPS	<4	<4	<4	<4
	CPS	<4	<4	<4	<4
	BCG/MAI/*M. smegmatis*	<4	<4	<4	<4
7	IFN-γ	<4	<4	<4	<4
	LPS	<4	<4	<4	<4
	IFN+LPS	<4	<4	<4	<4
	CPS	<4	<4	<4	<4
	BCG/MAI/*M. smegmatis*	<4	<4	<4	<4
10	IFN-γ	<4	<4	<4	<4
	LPS	<4	<4	<4	<4
	IFN+LPS	<4	<4	<4	<4
	CPS	<4	<4	<4	<4
	BCG/MAI/*M. smegmatis*	<4	<4	<4	<4
14	IFN-γ	<4	<4	<4	<4
	LPS	<4	<4	<4	<4
	IFN+LPS	<4	<4	<4	<4
	CPS	<4	<4	<4	<4
	BCG/MAI/*M. smegmatis*	<4	<4	<4	<4

[a]Each data point represents mean of triplicate samples, obtained from different macrophage donor sources,

< 4 nMwas the minimum level of the sensitivity of chemiluminiscence assay.

[b]Conconavalin-A stimulated human PBMC supernatant (CPS).

[c]Monocyte-derived macrophages were pre-activated for 24 hours prior to sampling for NO, with the following concentrations of cytokines:-IFN-γ 1000U/ml, LPS 1μg/ml, IFN-γ (1000U/ml) + LPS (1μg/ml).

[d]Any of the mycobacteria added singly to the cell cultures or after pre-activating with the different cytokine regimens.

Appendix: 10.5 The effect of LNMMA on BCG growth in culture medium

	LNMMA(mM)					
BCG (CFU/ml)	**10**	**1**	**0.1**	**0.01**	**0.001**	**0.0001**
10^4	+	+	+	+	+	+
10^6	+	+	+	+	+	+

(+) Presence of mycobacterial growth.

This table illustrates the effect of LNMMA on BCG growth. BCG at two different concentrations, i.e. 10^4 and 10^6 were incubated with various dilutions of LNMMA for 24 hours. Bacterial cultures were then harvested and semi-quantitative assessment of BCG was determined by visualising the microcolonies after 14 days.

Appendix 10.6 The effect of Conconavalin-A on BCG growth in RPMI-1640 medium

Con-A (μg/ml)	[^{3}H]uridine incorporation[a]	% Δ[b]
0	[c]128240+/-14406	0
20	102020+/-3345	-21[d]
40	67025+/-3351	-48

[a]Expressed as counts per minute (cpm).
[b] Percentage change in bacterial growth =
[1-cpm in the presence of Con-A/ cpm in the absence of Con-A] x 100
[c]Data represents mean of quadruplicates +/- SD
[d] Growth reduction (-).

This table illustrates the effect of Coconavalin-A on BCG growth in culture broth. BCG cultures at a concentration of $5x10^5$/well were plated out in 96-well plates in RPMI-1640 medium, and incubated with graded doses of Con-A. The number of surviving bacteria at the end of assay was determined by radiolabelling with 2μCi/well [^{3}H] uridine for 48 hours .

Appendix 10.7: Family trees of multicase TB families

Family Numbers

1

2

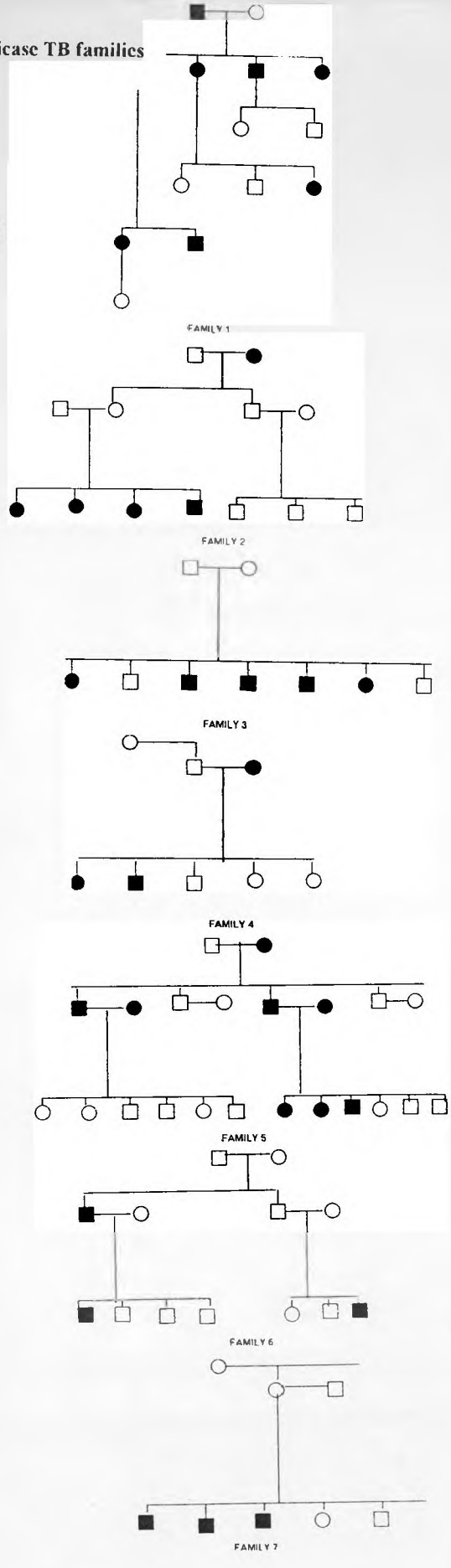
3

4

5

6

7



MALE

FEMALE

Infected = closed symbols

Un-infected = open symbols

Appendix: 10.8 Cellular studies on TB-infected patients from Family No: 1

One member of the Caucasian family with multicase TB was a 19yr old male who was initially diagnosed as being sputum positive in 1984 with a recurrence of the disease in 1990.

Following chemotherapy returned to in good health and had no other history of generalised impairment of his cell mediated immunity. This individual was selected for further analysis at the Clinical Immunology Laboratory, at the Medical School, in Birmingham and unless otherwise stated, all analyses were performed by Mrs. Pam Drysdale.
(Patient number x Family No: 1).

1] The ability of the patients phagocytic cells (polymorphs) to generate an oxidative burst was examined by the chemiluminescence and shown to be normal.

2] Lymphocyte surface marker analysis by FACS analysis revealed that the patient had a normal number of CD4 (helper) CD8 (cytotoxic) and γ/δ T-cells in the peripheral lymphocyte population. The polyclonal B lymphocyte population however showed an unusual expansion of a particular subset, the significance of which as yet cannot be explained or related to the patients apparent impaired cell-mediated immunity to TB infections.

3] Lymphocyte adhesion molecule expression (CD11, A, B, C, and CD18) were also found to be normal as were his lymphocyte proliferative responses to both PHA (Phytohaemoagglutination) and PPD (Purified protein derivative).

4] Limited cytokine analysis was also performed on the patients peripheral blood-derived lymphocytes by Dr. Rolf Keissling, of the Karolinska Institute in Stockholm.

The results show that the patients lymphocytes, when stimulated with the T-cell mitogen PHA, or with mycobacterial antigens (PPD) or heat killed whole MAI organisms, produced faster but similar cytokine responses to that of normal controls.

The profile of cytokines studied however included: IL1α, IL2, IL3, IL4, IL6, IL10, TNFα, IFN-γ, GM-CSF & G-CSF.

The results suggest that there does not seem to be any demonstrable deficiency of cytokine production in this individual although IL-10, and IL-12 were not tested as they were undefined this time. Further cytokine analysis therefore needs to be undertaken before a definitive statement can be made, ascribing a cytokine deficiency to the inceased susceptibility to mycobacterial infections in this patient.

5] EBV transformed B-cell lines from three infected family members, including patient number y were also generated as a renewable source of DNA to allow genetic analysis to be performed in Prof. Jennifer Blackwell's laboratory, at the Dept. of Medicine, Addenbrookes Hospital, Cambridge an attempt to look for genetic markers of susceptibility to mycobacterial disease. This analysis is currently onging in and the final results are still pending.

A more limited analysis was also performed on patient z , Family No: 1.

As with the previous patient outlined above, lymphocyte surface marker analysis revealed normal numbers of CD4+ and CD8+ cells in this patient also. Moreover, γ/δ T-cell numbers at 2% were also determined to be normal as were the patients B-cell numbers. The patients lymphocytes were also expressed normal levels of leucocyte adesion molecules CD11, A, B, C, and CD18.