BINDING OF *MYCOBACTERIUM TUBERCULOSIS* TO COMPLEMENT RECEPTOR TYPE 3 EXPRESSED IN MAMMALIAN CELLS: DEPENDENCE ON SERUM OPSONINS

by

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DECLARATION

I, ***Colette Cywes***, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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ABSTRACT

BINDING OF *MYCOBACTERIUM TUBERCULOSIS* TO COMPLEMENT RECEPTOR TYPE 3 EXPRESSED IN MAMMALIAN CELLS: DEPENDENCE ON SERUM OPSONINS

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Nonopsonic invasion of mononuclear phagocytes by *Mycobacterium tuberculosis* (*M. tb.*) is likely important in the establishment of a primary infection in the lung. *M. tb.* binds to a variety of phagocyte receptors, of which the mannose receptor and the complement receptor type 3 (CR3) may support nonopsonic binding. CR3, a β_2 integrin, is a target for diverse intracellular pathogens, but its role in nonopsonic binding remains uncertain. We have examined the binding of *M. tb.* to human CR3 heterologously expressed in Chinese hamster ovary (CHO) cells, thereby circumventing the problems of competing receptors and endogenously synthesised complement, which are inherent in studies with mononuclear phagocytes. The surface expression and functional activity of CR3 were confirmed by rosetting with beads coupled to anti-CR3 monoclonal antibodies (MAbs) and with C3bi-coated microspheres, respectively. We found that *M. tb.* binds 4-7-fold more avidly to CR3-expressing CHO cells than to wild-type cells, and importantly, that this binding is very similar in the presence of fresh or heat-inactivated human or bovine sera, or no serum. The binding of *M. tb.* to the transfected CHO cells is CR3-specific, as it is inhibited by

anti-CD11b and anti-CD18 MAbs; interestingly, binding is not inhibited by a MAb (2LPM19c) specific for the C3bi-binding site on CD11b. Electron micrographs of infected CR3-expressing CHO cells reveal the presence of intracellular bacteria enclosed in well-defined, membrane-bound vacuoles. We conclude that the binding of *M. tb.* to CR3 is nonopsonic and that the organism likely expresses a ligand that directly binds to CR3.

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TABLE OF CONTENTS

CONTENTS OF TABLES

CHAPTER SIX:

CONTENTS OF FIGURES

CHAPTER ONE:

CHAPTER THREE:

CHAPTER FOUR:

CHAPTER FIVE:

CHAPTER SIX:

ABBREVIATIONS

AFB	acid-fast bacilli
AIDS	acquired immunodeficiency syndrome
ALMϕ	alveolar macrophages
ATCC	American Tissue Culture Collection
ATP	adenosine triphosphate
BCG	Bacille Calmett-Guerin
bp	basepair
BSA	bovine serum albumin
C3	complement component 3
C3bi	inactivated portion of C3b
cAMP	adenosine-3', 5'-cyclic monophosphate
CD	cluster of differentiation
CFU	colony forming units
CFU-GM	colony forming unit, granulocyte-macrophage
CHO	Chinese hamster ovary
CHO-CR3S	CHO-expressing CR3 stably
CHO-CR3T	CHO-expressing CR3 transiently
CHO-MAC-1	CHO-expressing CR3 cells (Springer's cells)
CHO-WT	CHO-wild type cells
CMI	cell-mediated immune response

CR1	complement receptor type 1
CR3	complement receptor type 3
CR4	complement receptor type 4
CsCl	caesium chloride
CTL	cytotoxic T-lymphocytes
DMPD	dimethylpimelimidate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTH	delayed type hypersensitivity
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
ELAM	endothelial leukocyte adhesion molecule
ELDV	Glu-Ile-Leu-Asp-Val
ER	endoplasmic reticulum
FACS	fluorescence activated cell sorter
FcR	Fc receptor
FCS	fetal calf serum
FHA	filamentous haemagglutinin
FITC	fluorescein isothiocyanate
Fn	fibronectin
GA	glutaraldehyde
GMP140	granular membrane protein 140, P-selectin
HBSS	HEPES buffered saline solution

Hc	*Histoplasma capsulatum*
HI	heat-inactivated
HIV	human immunodeficiency virus
ICAM-1	intercellular adhesion molecule-1
IFN	interferon
Ig	immunoglobulins
IL	interleukin
kb	kilobase
kDa	kilodalton
L-J	Loewenstein Jensen
LA	Luria agar
LAD	leukocyte adhesion deficiency
LAK	lymphokine-activated killer
LAM	lipoarabinomannan
LB	Luria-Bernardi broth
Leu-CAMs	leukocyte adhesion molecules
LFA	leukocyte function antigen
LPG	lipophosphoglycan
LPS	lipopolysaccharide
M. tb.	*Mycobacterium tuberculosis*
MAb	monoclonal antibody
MDM	monocyte-derived macrophages
MDR	multi-drug resistant
MHC	major histocompatibility complex

MOPS	major outer membrane proteins
MSX	methionine sulfoximine
MTX	methotrexate
NRAMP	natural resistance-associated macrophage protein
OD	optical density
p	plasmid
PAS	para-amino salicylic acid
PBMC	peripheral blood mononuclear cells
pBS	pBluescript
PBS	phosphate buffered saline
PE	R. Phycoerythrin
PGL	phenolic glycolipid
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RGD	Arg-Gly-Asp
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
S-TBS	skim milk/Tris-buffered saline
S A	South Africa
SV40	Simian virus 40
TB	tuberculosis
TE	Tris/EDTA
TEM	transmission electron microscopy
Tris	tris(hydroxymethyl)aminomethane

v/v	volume/volume
VCAM	vascular cell adhesion molecule
VLA	very late antigen
VNR	vitronectin receptor
w/v	weight/volume
WBC	white blood cell
WHO	World Health Organisation
ZN	Ziehl-Neelsen

CHAPTER ONE

INTRODUCTION

Tuberculosis (TB) has claimed millions of lives in past centuries throughout the world, with no real sign of abating in the near future, making the causative agent, *Mycobacterium tuberculosis*, one of the most successful human pathogens known to man. Presently one third of the world's population is infected with *Mycobacterium tuberculosis*, and of these more than 50 million people harbour drug-resistant strains. It is predicted that thirty million people will die from TB in this decade alone (WHO 1995). Twenty-six percent of all adult deaths in the developing world are due to TB, and every year almost 300 000 children die of the disease. Moreover, in AIDS TB is one of the leading causes of death.

These astounding statistics seem incredible, but they are a testament to the organism's remarkable infectiousness: every year a single untreated person infects 10 to 15 people, in each case often via a single cough carrying 1-3 bacilli.

1.1 History of Tuberculosis:

For many generations the origins of TB and its effects on various populations and cultures have been the topic of widespread research and discussion. How and why the now- curable disease continues to maintain such a high profile in many countries is not well understood, but the fact that these epidemics are accompanied by poverty, overpopulation and poor socio-economic standards is widely accepted. When

reviewing the literature on the history of TB, it is evident that the complete story may never really be known, as available evidence suggests that TB predates written documentation. Useful records that may have been produced by earlier communities are largely lost or destroyed and are therefore sparse. Such early documentation may have described not only the origin of the disease, but also the subsequent effects on community growth and development.

Early indications of TB-like disease occurring during the Neolithic period have given rise to the debate whether *M. tb.* originated from *M. bovis* or *vice versa;* the Neolithic was the period in which man began to domesticate animals, such as cattle in the Mediterranean (5000-7000 BC) and the Middle East (4000-5000 BC) and elephants in India (before 2000 BC). It was from one of the early domesticated Indian elephants that the first account of animal-related TB was documented (Manchester, 1984).

As far as can now be determined, the history of human TB is thought to have originated in Egypt between 3700-1000 BC, with suggestive skeletal discoveries, but care must be taken not to assume that all skeletal deformities were the result of *M. tb.*, as *M. bovis* and non-tuberculosis spondolytis may also have been responsible. A Neolithic skeleton discovered in Heidelberg, Germany dating ± 5000 BC, has been thought to show tuberculosis lesions, but this is still hotly debated (Metcalf, 1991). Definitive evidence for ancient TB was first obtained from an Egyptian mummy of a 5-year-old child from the Dynastic period ± 3400 BC, from which acid-fast bacilli (AFBs) were obtained from the lungs and spine. A young male mummy from the Bronze Age in Jordan (3150-2200 BC) revealed similar bacilli-filled abscesses and organs, suggesting that TB initially originated in the near East (Salo etal, 1994) .

In comparison with these findings, northern Europe and the United Kingdom have been infected only recently with *M. tb.*, as the earliest findings of skeletal TB occurred in Denmark with a skeleton dated 2500-1500 BC. Britain's first evidence of TB dates to 3-5 AD, with the Americas thought to follow when explorer Columbus landed in America in 1492 (Snider, 1994). A later theory that the Vikings may also have contributed to the establishment of TB in America is becoming more popular, as is the possibility that TB spread from South America to North America, as suggested by the recent discovery of a Nazca culture mummy child (700 AD), discovered in Southern Peru (Metcalf, 1991).

As art was often used as a historical record in earlier civilisations, it may be deduced from the many hunchbacked figures adorning tombs and pyramid walls in Ancient Egypt during the Dynastic period before 3400 BC that TB affected Egyptian civilisation. Similar artistic interpretations of the humpbacked Kokopelli rain-priest from Central and Southern America, in the Pueblo and Inca cultures, are suggestive of TB in these communities as well, which predate the recent discovery of the Nazca mummy (Wellmann et al., 1970; Morse, 1967).

Written documentation tends to follow the same distribution as that described above, with the first documented evidence originating from an Egyptian medical papyrus, in which TB and its symptoms are described. The first Asian documentation of pulmonary TB described it as 'lung fever" and 'lung cough" in the early Chinese writings of ± 2700 BC and later in the Rega-Vedas hymns of the Hindu Indo-Ayran civilisation in 1500 BC (Morse, 1967; Metcalf, 1991; Snider, 1994).

The earliest Greek literature describing consumption were from the early works by Homer in 800 BC, followed by a detailed description of 'phthisis" by Hippocrates in *de*

Morbis (500 BC). Aristotle was the first to suggest the contagiousness of "phthisis" (384-322 BC), with later reports by Galen in 131-200 AD substantiating Aristotle's belief. Plautus, 184 BC, was the earliest Roman to document TB, followed by many accounts of TB-like infections during the Byzantine era (Metcalf, 1991; Snider, 1994).

This evidence suggests that TB has been widespread for many millennia, with its origin possibly in the near East, which supports the theory that the domestication of animals and social civilisation led to the development of TB.

More recent studies of TB began in the Renaissance, when Girolamo Fracastora (1483-1553) re-documented the contagiousness of TB, and Franciscus Sylvius (1614-1672) described the characteristic tissue nodules as 'tubercules". This was followed by the historic experiments of Pasteur in 1862, which suggested the airborne nature of the bacilli; but it was Robert Koch's physical evidence, with his isolation of the *M. tb.* bacillus in 1882, which convinced the medical profession that TB was the result of a bacterial infection and therefore clearly transmissible (Morse, 1967; Snider, 1994).

Subsequent documentation of the spread and incidence of TB was as infrequent and inaccurate as in earlier centuries. Over- and under-reporting of TB, due to the many other wasting diseases of those eras, and the stigma associated with TB, hindered the collection of accurate epidemiological data. It was only in the late 19th century, when compulsory notification of the disease was initiated in New York in 1897, followed in the 1900s by Norway, Cape and Natal colonies of South Africa, Denmark, England and Scotland, that more realistic statistics were obtained (Metcalf, 1991).

The epidemic proportions that alarmed communities around the world were thought to have originated in Western Europe and Northern America during the rapid

industrialisation in the 19th century, after which the disease spread to Eastern Europe (Snider, 1994). Sub-Saharan Africa seems to have been spared the past epidemics, probably due to its relative isolation, but this has resulted in the current epidemic sweeping the continent, as discussed later in this chapter.

1.2 Conditions Influencing the Occurrence of Tuberculosis:

Increases in population, urbanisation and later industrialisation were major determinants in the progression of the disease. Industrialisation brought joblessness and poor health conditions, including poor diet and lack of sun exposure, due to the long work-days indoors. This together with the overcrowding in the cities led to widespread disease and increased mortality rates. Migration to cities to find work and then back into the country or to other cities, associated with the lack of work, helped to spread the disease from one geographical point to another and probably served to infect otherwise unexposed individuals. During this period, documentation of the environmental modifying effects on disease progression by Dr Ernest Livingstone Trudeau, led to the development of the first sanatoria for the treatment of tuberculosis, with exposure to high altitudes, dry air and sunshine, thereby serving to spread the disease as far afield as the Swiss Alps, Rocky mountains in America, Australia and South Africa. Colonisation of the New and Old World by European invaders served as a catalyst for the initiation and spread of TB amongst indigenous populations. The exploitation of their land and natural lifestyle led to impoverishment of the local populations and produced overcrowding and severe shortages of food in the settlements, orchestrating the ideal niche for the TB bacillus to thrive and make full use of the absence of innate immunity. The controversial suggestion that racially

determined genetic differences account for a higher susceptibility to TB amongst Blacks has long been debated. Genetic variation, as opposed to increases in disease due to changes in social standards and environment, has been the subject of many studies, but this proposition has not been established beyond reasonable doubt, and therefore remains speculative (Metcalf, 1991; Snider, 1994).

The frequent occurrence of war in the Western world has done little to stem the disease or the spread thereof. The common theme of overcrowding and decline in living conditions and nutrient intake, which associates with the spread of TB epidemics, is surely one of the most destructive and longest enduring features of any war, as highlighted by the TB mortality figures in England during the First and Second World Wars (Figure 1.1) (Metcalf, 1991).

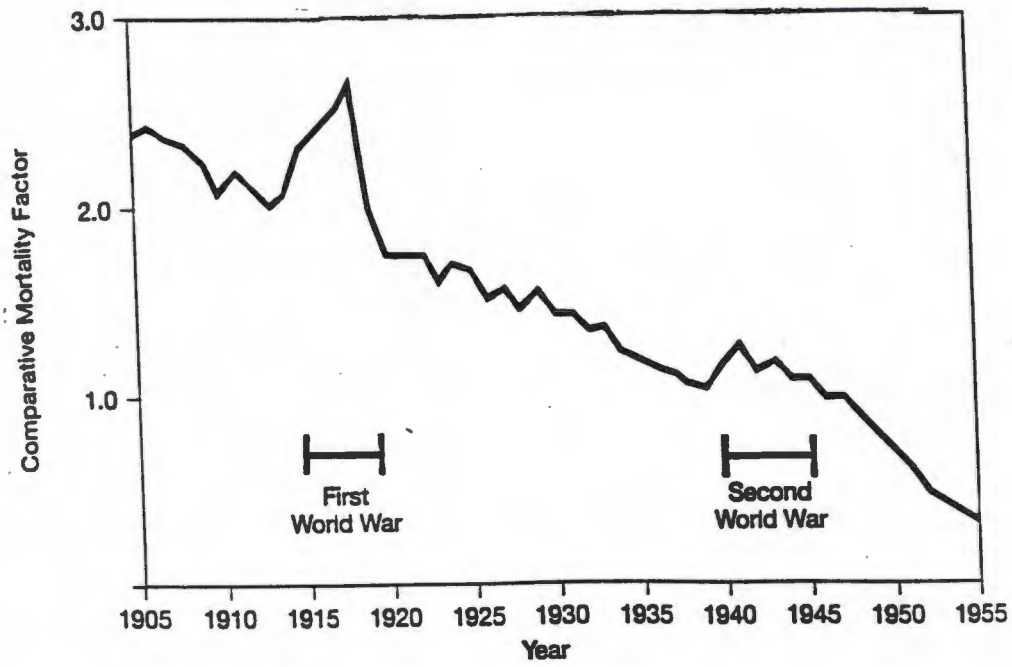
1.3 Past Treatments of TB:

Prior to the development of chemotherapy in 1910, a few early abortive attempts to develop a protective agent for the eradication of the infection were initiated by Koch. The pre-chemotherapeutic treatments of tuberculosis, although not as powerful as drugs, have provided the medical profession with insight into the disease and its progression, making it the cornerstone of current therapy.

Initial documentation and treatment of "phthisis" by Hippocrates, the father of modern medicine, revolved around his humoral theory, which entailed resection, emetics and purgation, as well as the use of leeches, to correct the altered state of the four humours (body fluids, namely blood, phlegm, yellow and black bile) thought to be instrumental in the disease process. Similarly, Thomas Sydenham treated London consumptives in

Figure 1.1: Effects of World Wars I and II on the tuberculosis mortality rate among the English and Welsh during 1905-1955

(Source: Coovadia, H.M. and S.R. Benatar. A Century of Tuberculosis: South African Perspectives. 1991. Oxford University Press)



Comparing the mortality factor for respiratory tuberculosis, using the incidence of tuberculosis during 1938 as the standard (=1). This figure depicts the increases in deaths due to tuberculosis during the World Wars I and II as compared to the pre- amd post-war periods.

the 1600s with pectorals and sugar liquorice tablets, to try and remove the 'hot and acrid blood" caused by the sulphur and fumes breathed in by the factory workers. Francois Broussais (1772-1838) enforced starvation, purgation, venesection and leeches on his patients, with the thought that the disease originated in the gut via the ingestion of toxins which in turn irritated the organs. G-L Bayle (1774-1816) and later R.T. Laennec (1781-1826) were the first to move away from the humoral theory and depicted the disease as an organ-specific disease, as deduced from their multiple autopsies, probably established without prior inflammation.

Hermann Brehmer (1826-1889) pioneered the first Sanatoria in the German mountains, to enforce the current theories of his colleagues, which entailed high altitudes and exercise to strengthen the heart, rest in fresh air and later hyperalimentation. The well documented Davos in the Swiss Alps started the sanatoria movement in the rest of Europe, as described in Thomas Mann's famous *Magic Mountain.* In the late 19th century, Dr E. Livingstone Trudeau's documentation of the environment's modifying effect on the development of the disease led to the start of the sanatorium era in the USA. His involvement in research into the immune function of these individuals earned him world-wide respect and acknowledgement.

The close patient-doctor interactions developed in these sanatoria allowed the first specific treatments for tuberculosis to be instituted. This invasive procedure known as artificial pneumothorax was developed by Carlo Fornalini in 1894, based on experimental data describing the elasticity of animal lungs produced by James Carson (1772-1843) of the UK. This was the first procedure to show specific clinical improvement for tuberculosis patients. An extension of this procedure developed by John B. Murphy in 1899, known as 'Murphy's button", relied on anastomosing the

hollow abdominal viscera, forcing the lung to rest with co-cicatrization of the tubercle foci. These direct interventions served to reduce the high mortality rate of TB and were widely used for many years.

In South Africa, a variation of Sauerbach and Schepelman's paralysis and raising of one diaphragm was developed, in which not only one but both diaphragms were raised, thereby reducing the risk of air embolism experienced with conventional acute pneumothorax. Permanent collapse of the lung (thoracoplasty) in which the 5-7th ribs were removed and the lung resected from the lung apex, to allow concentric collapse, was shown to reduce sputum positivity dramatically, and therefore frequently used in South Africa among the White population, but not for Black TB patients as it reduced their capacity for physical labour.

It was only with the development of Streptomycin in 1943 by Albert Schatz and Selman Waksmann, from the soil actinomycete *Streptomyces griseus*, followed by the discovery of para-amino salicylic acid (PAS), by Jorgen Lehmann in 1944 and later the development of Isoniazid in 1952, that heralded the start of effective treatment of the disease in the New World. Although these drugs initially served to stem the disease, it also spurred the development of mutations within the bacilli, resulting in the recent identification of multi- drug resistant (MDR) strains of TB, which are selected for by the inappropriate administration of chemotherapeutic agents (Dubovsky, 1991).

1.4 The Global Effect of Tuberculosis:

Inaccurate, or complete lack of, documentation of TB and TB-related cases has made the determination of the incidence of TB problematic, but as stated previously, it is

estimated that annually 8 million new cases, of which 95% occur in the Developing World. The highest incidence of death affects the most productive age group of the population, namely individuals between the ages of 15 and 59 years of age, serving to economically cripple developing nations (Snider, 1994; WHO 1995). As seen during the decades of disease in the New World, TB is closely linked to the demographic, historical, financial, political and environmental changes in the world, making industrialisation, wars, mass urbanisation and over-population key determinants of the disease process and progress; all factors that are affecting the Sub-Saharan countries at present.

Recent upsurges of TB in the Western world have brought home the realisation that TB will not be eradicated by 2010, as was predicted during the planning of the USA health strategies in 1989. Unemployment, immigration of people from high incidence countries to the Western world and the world-wide explosion of HIV and drug abuse have helped to undermine the effectiveness of TB control programs. These factors together with the inability to treat MDR TB have once again served to make TB a major threat to public health in both developed and developing countries.

1.5 The South African Experience:

TB in South Africa (SA) is an excellent example of the effects that socio-economic conditions, politics and demographics have on the incidence and spread of TB. Little if any information is available regarding the existence of TB in pre-colonial SA, other than the reports made by missionaries and travellers to remote parts of Africa before the colonisation of the Cape by the 1652 Dutch settlers, who used the Cape of Good

Hope as a trading station for the ships travelling to the East. Thus until the 17th century, few episodes of TB were thought to exist south of the equator. Once European colonisation started, and native people were exposed to the bacillus, the disease spread quickly, with the first documented case of TB identified in late 18th century, signifying the spread of TB to the locals. Dr John Polsen recorded that no TB was seen in the Bantu tribes in the late 19th century, but that in contrast the ethnically distinct Khoikhoi (Hottentots) and San (Bushmen) had suffered severely; it is significant that the latter two populations had the earliest and most extensive contact with the European sailors. Controversy still exists whether TB had existed on a low level in Blacks prior to colonisation, and Dr Peter Allan, who has researched the topic extensively, suggested that there was evidence of endemic TB in the Ciskei and Transkei, as well among the Zulu's of Natal, before colonisation, but certainly not of the epidemic proportions encountered after colonisation. It was only after the turn of the century with the introduction of health statistics and death registration that the extent of the racial and geographic distribution of TB was realised.

As with the industrialisation of the Western world, the development of the mining industry in South Africa in the late 1800s is closely associated with the extensive migration of thousands of Blacks as cheap labour from within South Africa's borders and from neighbouring African states, which initiated epidemic proportions of pulmonary TB amongst this population. Similar to the industrialised countries, the miners spent long hours underground, in poorly ventilated, confined spaces with hundreds of others, and were accommodated in close, overcrowded living conditions, with poor socio-economic standards and nutrition. This helped to lower their immune status and enhance the incidence and spread of TB amongst this community. The

repatriation of sick workers back to their rural homes served as the perfect vehicle for the dissemination of the disease to the indigenous rural populations not yet in contact with the colonials. In 1912, the incidence of TB in rural communities could be traced directly to the number of migrant workers in the community.

A further influence on the spread of TB in SA was the extensive advertising campaign for SA as a health resort for "ailing consumptives" from Europe in the late 1800s. Most of these resorts were in the Northern Cape, but all the passengers disembarked in Cape Town en route, helping to introduce and spread the disease further. The development of urban centres, with greater job opportunities, attracted large numbers of rural Blacks to towns and cities, thereby exposing them to TB, which was compounded by the overcrowded, poor socio-economic housing conditions similar to those on the mines, making TB three times more prevalent in urban than rural areas. These housing areas were fast classified as slums and the introduction of the Slums Clearance Act of 1934 served to force large populations of Black and Coloured people to the outskirts of cities, thereby providing false statistics of the subsequent decrease of TB in these areas. The racially based political constraints introduced after the declaration of SA as a Republic in 1963 served to keep Blacks in confined areas, both in the work place and at home, that were of a poor economic standard and aided the spread and virulence of the disease. Subsequently, with the abolition of the immigration (pass) laws in the 1970s, large numbers of Blacks sought work in the cities, emigrating from areas of high TB prevalence. This rapid urbanisation by Blacks in Cape Town and the Western Cape region coincides with a remarkable increase in the incidence of TB among the Coloured population, who are the traditional residents of this region. Indeed this has resulted in a new epidemic of astounding proportions, with

Figure 1.2: Age-, gender- and ethnic-group incidence rate of tuberculosis in South Africa in 1993

(Source: Epidemiological Comments. 1993. Department of Health, Republic of South Africa)

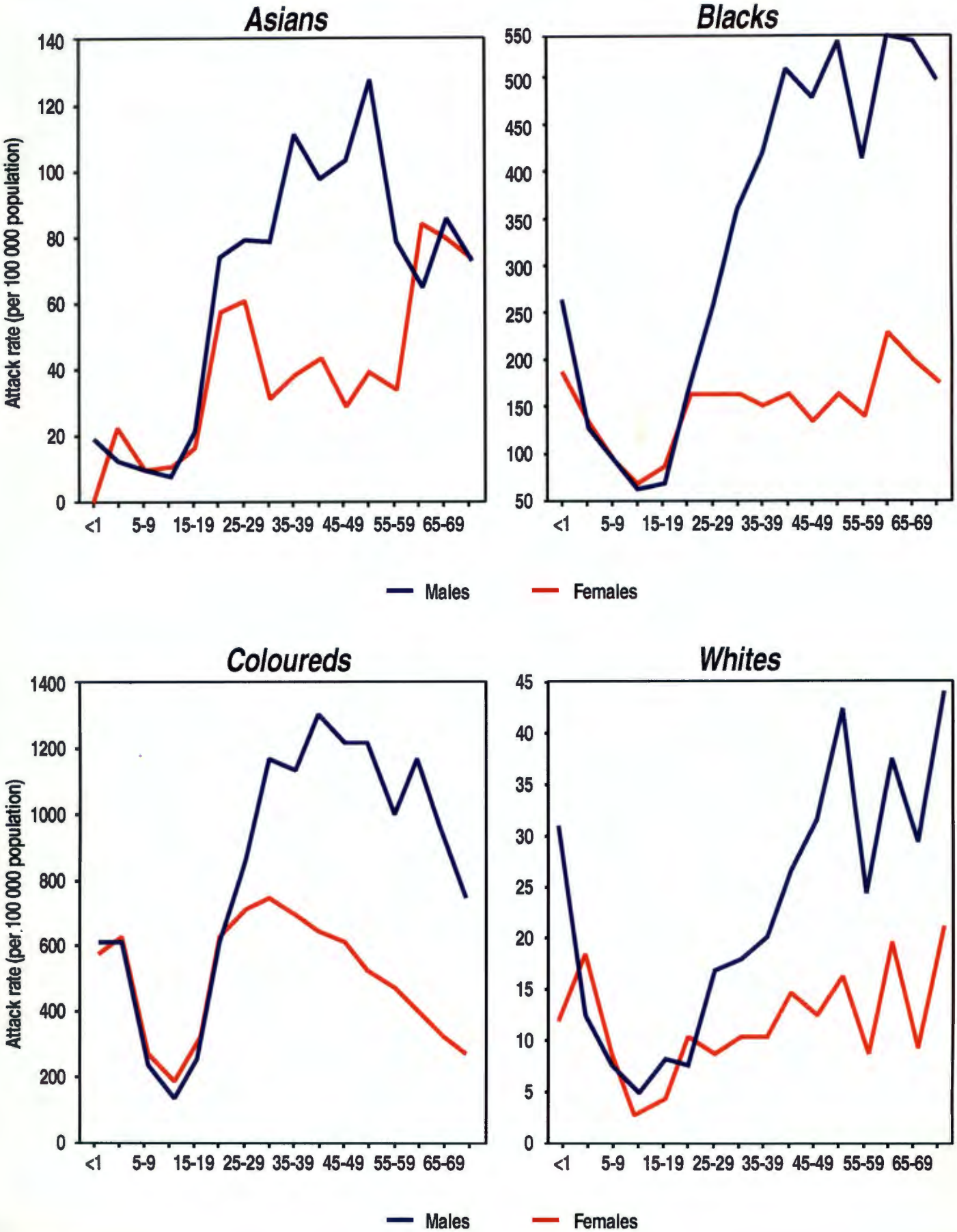
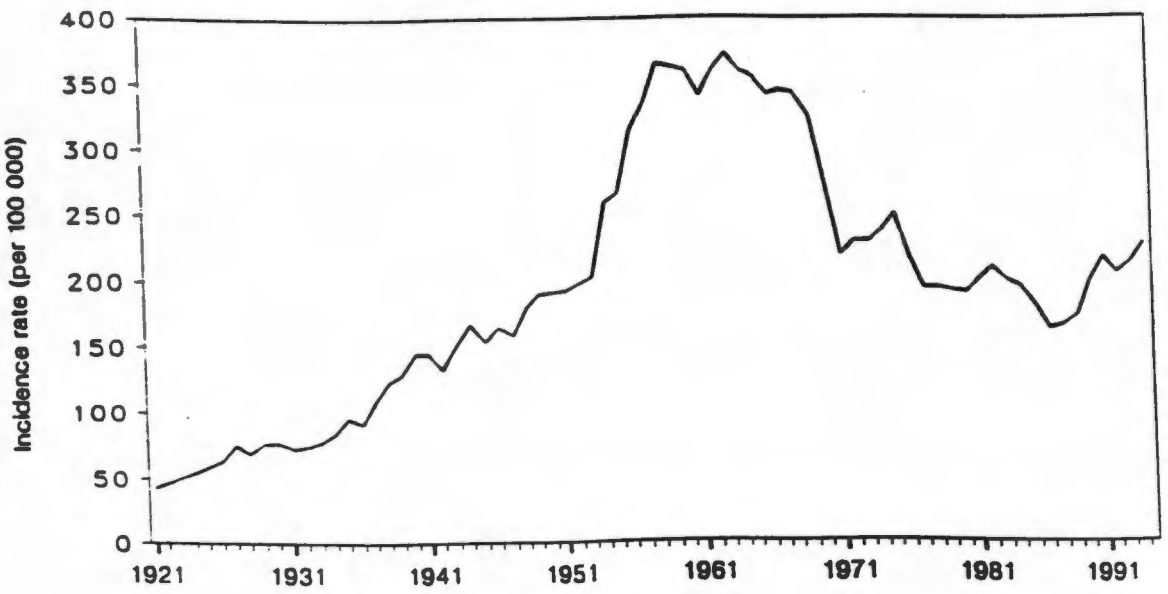


Figure 1.3: Historical perspectives of the tuberculosis incidence rate in South Africa between 1921 and 1993

(Source: Coovadia H.M. and S.R. Benatar. A Century of Tuberculosis: South African Perspectives. 1991. Oxford University Press)



Tuberculosis increased steadily in South Africa from 1921 to peak during the mid-1960s, with a sharp decline after the introduction of chemotherapeutic agents in the late 1960s.

incidence rates in home communities in the Western Cape exceeding 1000 per 100 000 (Metcalf, 1991; Packard, 1991; Strebel and Seager, 1991).

1.5.1 South African Statistics:

It is estimated that 6-10 million South Africans are infected with *M. tb.*, with approximately 60 000 new cases notified and 6 000 registered deaths per annum. It must be realised that, as in the past, the notification and registration of infections and disease are often far below real values, due to the lack of education and primary health care in the rural areas of South Africa. Adding to this problem are the continual influxes of refugees and people from North Africa, often infected with TB, which adds to the inaccuracy of both the population statistics and the infection incidences for our country. Migration between provinces within SA also cause confusion and lack of accurately documented data (Packard, 1991; Strebel and Seager, 1991).

Although most South African children are inoculated at birth with BCG to protect them from the high incidence of TB meningitis, figures for childhood tuberculosis are still high, 0.3/100 000 in children below 5 years (Strebel and Seager, 1991). Interestingly, there seems to be no difference in the incidence of TB between boys and girls below the age of 10 (Figure 1.2), but a 9% increase is recorded in young males between the ages of 12 and 19 years, suggesting an increased susceptibility during puberty, probably related to the growth spurt accompanying this period. As indicated in Table 1.1, the highest incidence of TB seems to occur between the ages of 1 and 4 years, with an incidence of 240.8/100 000, in 1994, as compared to 164.95/100 000 in 1992, with a similar incidence occurring again in adulthood, 215.18/100 000, with

pulmonary TB featuring predominantly in all age groups. Figure 1.2 indicates clearly the age-, gender- and group-specific distribution of tuberculosis in SA for 1993, highlighting two peaks of TB, during puberty and then again in adulthood for males. As expected, TB is most prominent within all race groups in old-age, as the older individual's immune-system is not able to deal with the bacterial load, making old-age homes and frail-care hospitals a prime target for TB epidemics (Epidemiological Comments, 1993).

Judging by the trends indicated in Figure 1.3, the TB epidemic seems to have peaked in SA in the 1960s, with approximately 350 cases reported per 100 000, predominantly in the Black population. In 1994, it is interesting to note the increase in the TB rate was 2-fold greater in Indians, 11-fold greater in Blacks, and a stunning 43-fold greater in the Coloured population, as compared to the incidence in Whites for the same period in 1993 (Table 1.2). Although this increase in the incidence of TB has continued over the last decade, the incidence of TB in the Coloured population has sky-rocketed from 633/100 000 in 1987, to a current incidence of 705.86/100 000 for 1994, suggesting a shift in the affected population group rather than an eradication of the disease (Table 1.3) (Epidemiological Comments, 1995).

The apparent shift in TB from the Black to the Coloured populations is reinforced by the data in table 1.4 highlighting the geographical distribution of TB through the country, as the greatest percentage of the Coloured population resides in the Western Cape, where the incidence of TB is the greatest, at 711.3/100 000.

The TB mortality rate in South Africa is one of the highest in the world, with 2408 individuals dying during 1994, and 80 609 new cases reported for the same period (Epidemiological Comments, 1995).

Table 1.1: Age-related Incidence of Tuberculosis in South Africa in 1994

AGE (years)	POPULATION (X1000)	PTB	TOTAL TB	INCIDENCE (per 100 000)
< 1	1058	1172	1794	169.56
1 - 4	4075	5887	9813	240.8
5 - 14	9197	8028	10662	115.93
15 +	26232	53870	56446	215.18

(PTB - pulmonary tuberculosis)

Table 1.2: Ethinic Distribution of the Incidence of TB in 1993 and 1994 in South Africa

POPULATION GROUP	INCIDENCE / 100 000 1993	INCIDENCE / 100 000 1994
Asian	50.78	38.47
Black	206.58	173.4
Coloured	712.53	705.86
White	18.75	16.3

Table 1.3: Ethnic Distribution of TB in South Africa for the Period January to December 1994

ETHNIC GROUPS	POPULATION (X1000)	PTB	TOTAL PTB	INCIDENCE (per 100 000)
Asian	1037	381	399	38.47
Black	30902	48393	53586	173.4
Coloured	3446	19854	24324	705.86
White	5177	665	844	16.30

Table 1.4: Geographical Distribution of TB in South Africa in 1994

PROVINCE	POPULATION (X1000)	TOTAL TB	INCIDENCE (per 100 000)
Western Cape	3589	25530	711.2
Eastern Cape	6517	14961	229.5
Northern Cape	762	3257	427.4
Free State	2813	13567	482.3
KwaZulu/Natal	8597	6510	75.72
Eastern Transvaal	2918	2251	77.14
Northern Transvaal	5115	1686	32.96
Gauteng	6886	9703	140.9
North-West	3369	3004	89.16
R.S.A. Total	40562	80469	198.4

1.6 Pathogenesis of Tuberculosis:

Tuberculosis represents a continual struggle between bacilli with a remarkable capacity for long-term persistence, and the host immune system which attempts tosuppress or even eradicate their growth. The outcome of this struggle depends not only upon the dose and strain of the invading bacteria, but also on the ability of the host to respond quickly and efficiently to the invasion. There are a number of well documented outcomes of a primary *M. tuberculosis* infection. First, many individuals are exposed, but successfully eliminate the pathogen, so that only a delayed hypersensitivity response remains upon skin testing. Second, the infected individual develops a calcified lesion, visible on radiological examination, but suffers no symptoms of the disease, suggesting an efficient cell-mediated response (CMI) and containment of the bacilli. This explains why 1/3 of the world's population is infected with TB but only 30 million have active tuberculosis, or why only 5-10% of infected individuals develop fulminant TB during their lifetime, suggesting the development of protective immunity (Ehlers, 1995). Third, the infected individual requires a longer time to respond to the invasion and can only limit disease spread once the bacilli are shed into the bloodstream and lymph, where they are eradicated by a local immune response, after which the infection is stabilised. Fourth, the individual is unable to arrest bacillary growth, with liquefaction of the granulomatous lung lesions and dissemination of the disease throughout the body, establishing systemic tuberculous disease (Dannenberg and Rook, 1994).

One of the most accepted theories of the pathogenesis of TB is that it represents a balance between the bacilli's ability to multiply and the host's ability to respond

appropriately. There are two ways in which the host can respond and staunch the bacterial growth: first, by damaging the cells in which the bacilli initially find themselves, i.e. the unactivated alveolar macrophage, and thereby exposing the bacilli to an unfavourable extracellular environment; and second, by evoking a CMI that recruits activated monocyte/macrophages, which will phagocytose the extracellular bacilli and contain and attempt to kill the bacilli intracellularly. The balance of these two responses will dictate the nature of the disease and the extent of the tissue damage, as they are both accompanied by varying levels of inflammatory events involving clotting factors, enzymes, cytokines, nitric oxides and oxygen radicals.

Much of our understanding of the pathogenesis of TB has originated from the Bowden and Lurie studies in inbred, and therefore susceptible, rabbits, which seem to develop TB in much the same way as humans. In this model, resistant rabbits depict adult humans, whereas immune-compromised individuals and infants correspond to the susceptible, inbred rabbits. On studying the disease process in this model, Dannenberg has identified four stages, which are widely accepted as representative of the human disease, namely stage 1, the stage of no bacillary growth; stage 2, the symbiotic stage; stage 3, the cell-mediated and delayed-type hyersensitivity immune response; and stage 4, the liquefaction of the caseous center (Dannenberg, 1991).

The infection is initiated by the inhalation of *M. tb.* bacilli, usually as a clump of between 1 and 3 bacilli (1 unit), probably in the form of a droplet of 5μM in diameter, which is able to pass unhindered through the bronchial passages into the mid-lower zone of the subpleura, where there is a high oxygen tension, and where the bacilli are rapidly phagocytosed by resident alveolar macrophages. The alveolar macrophage is a

resident macrophage of the lung, which is continually stimulated by small particles of dust, carbon and other organisms that are inhaled, contributing to the fast removal of particles from the alveolar environment. These cells are part of the first line of defence in the lung and must contain many potential infections, which thereby remain subclinical; however, their ability to kill invading pathogens is variable. Lucas (1994) suggests that upon initial infection (primary infection) with *M. tb.*, the alveolar macrophages carry the bacilli to the nearby lymph nodes, thereby establishing a second focus, distinct from the local lesion, which may explain the lymphadenopathy accompanying early TB; moreover, all patients undergo a bacteremia at this stage, in which bacilli are potentially culturable from the blood and urine.

Once the initial infection is established, the bacilli multiply within immature host macrophages, which have been recruited from the bloodstream and bone marrow by the cytokine response, lyse the cell, and are again phagocytosed by newly recruited monocyte-macrophages. These newly recruited macrophages are unable to contain and kill the bacilli, but allow the formation of granulomas, with bacilli surrounded by recruited macrophages. No tissue destruction occurs at this stage, as the host has not yet become hypersensitive to the tuberculin toxin secreted by the invading pathogen. Cytokines are continually being secreted by both the damaged cells and the recruited cells, eventually leading to the recruitment of the T-lymphocytes, which will serve to activate the incoming macrophages and allow intracellular killing to occur. At this stage a major cell mediated immune response is established in the immune-competent host.

At this point, two to three weeks post-infection, the bacilli are in log phase and the host has become tuberculin-positive. Due to hypersensitivity to the tuberculin toxin

from the bacilli, necrosis of the granulomatous lesion may now take place, suggesting a toxic reaction between host and bacilli, leading to destruction of local tissue. This destruction of the tissue enables the host to inhibit bacillary replication, because of the unfavourable conditions created by the high fatty acid content, low pH, and anoxia accompanying caseous necrosis. The success of containment of the bacilli at this stage depends upon the multiplication rate of the bacilli - an expression of bacterial virulence - and the efficiency of the host to limit bacterial growth. The cytokine-activated macrophages surrounding the granuloma are able to phagocytose and inhibit growth of the bacilli, and may be able to kill them.

Cytokine activation of macrophages is a component of T-cell mediated hypersensitivity, which develops as the T-lymphocytes are exposed to mycobacterial antigens presented to them by the infected macrophages and possibly other antigen-presenting cells. To enable an effective immune response, bacterial proteins are presented to the host immune system in the context of either major histocompatibility complex class I (MHC I) or MHC class II (MHC II) proteins. Classically, the p[resentation of MHC I-associated antigens has been thought to initiate cytolytic activity mediated by CD8 T cells, as in the case of viral infections. In contrast, the MHC II response comprises of CD4 T cell enhancement, which usually accompanies protozoan and bacterial infections (Kaufmann, 1995; Marrack and Kappler, 1994). In some bacterial infections, such as TB, the microbial localisation may dictate the antigen processing pathway or the entire peptide may be required and not only the epitopes, for T cell recognition, suggesting that more than just one T cell population may be involved in the immune response (Kaufmann, 1995).

Responder T helper cells react by secreting cytokines to activate local macrophages to ingest *M. tb.* as well as to recruit activated macrophages from the bloodstream, which may enable the macrophage to contain or even kill the bacilli. However, the lack of *in vitro* evidence that human macrophages can actually kill *M. tb.* has caused much controversy, leading to a modification of our concept of protective immunity. The cellular immunity is dependent upon the secretion of inteferon-gamma (IFN-gamma) and the recruitment of both CD4 and CD8 T cells to effectively contain and eliminate the infection. The subpopulations of T cells involved in the protective immunity during tuberculosis has long been debated, and current theory suggests that a subpopulation of $CD44^+$ and $CD45RB^-$ CD4 T (Th1) cells develop in response to *M. tb.* (Griffin et al., 1994), which are responsible for the secretion of IFN-gamma, IL-2, and TNF-α in response to *M. tb.* antigens, Ag85B and low molecular weight peptides, ESAT-6 and ST-CFP (Marrack and Kappler, 1994; Boesen et al., 1995; Porcelli et al., 1992).

A second subset of T cells recently recognised as $CD4^-CD8^-$ $\alpha\beta$ T (Th2) cells respond to mycolic acids and lipoarabinomannan commonly found in *M. tb.* and *M. leprae*, respectively, that enable the host to induce T cell proliferation, via induction of IL-10 and IL-4 secretion, and lysis of infected cells via the CD1b receptor (Kaufmann, 1995; Cooper and Flynn, 1995), as well as the stimulation of B cells to produce antibodies to the bacterial antigens presented (Marrack and Kappler, 1995). These cells are thought to develop as part of the early innate response to infection (Beckman et al., 1994).

A third T cell population consists of $\gamma\delta$ T cells, whose role in *M. tb.* infection is thought not to be crucial in the initial containment of the bacilli, (although they are able to respond to non-proteinacious phosphate-containing *M. tb.* antigens), but rather to

respond to the increase in IL-2 and IFN-gamma preceeding the Th1 CD4 response (Cooper and Flynn, 1995).

A Th2 CD4 T cell response has shown not to be enhanced systemically or at the site of pathology in humans, unlike in *M. leprae*; the Th1 response together with the CD8 T cell proliferation are more apt to contain and eliminate a primary infection. The direct role of CD8 T cells, although still controversial, has been recognised to transfer immunity and cytotoxic specificity to *M. tb*. infected macrophages (Lin et al., 1996). It is now proposed that cytotoxic T-lymphocytes (CTL), as well as helper T-lymphocytes and macrophages, are recruited to the site of infection, but it is the CTLs that recognise and lyse the incapacitated, infected macrophage, thereby releasing the bacilli to: (1) expose the bacilli to toxic radicals secreted during the lysis of the macrophage; (2) expel the bacilli into the unfavourable necrotic environment of the granuloma focus; or (3) expose the bacilli to fresh, highly activated MDMs that are better able to contain ingested *M.tb* (Kaufmann, 1995).

At three weeks post infection, if the lesion is not calcified and contained, the susceptible host will allow the bacilli to escape the necrotic focus and continue growth in the surrounding macrophages. This together with increased tissue destruction and an inability to contain bacillary growth will lead to an increase in the size of the lesion and possible erosion through the tracheobronchial walls into surrounding lymph nodes and blood vessels, by which the bacilli will be efficiently disseminated throughout the body, eventually resulting in death. In the resistant host, a strong CMI develops, which will aid in locally activating macrophages, which will in turn contain the spread and replication of the bacilli. The increased cytokine production, and T-cell activation enhances the recruitment and activation of macrophages. A dense macrophage layer is

therefore formed around the caesous centre, walling off the lesion and arresting the disease process. If bacilli should escape the mantle of protection, the CMI response will be evoked efficiently and the bacilli inhibited wherever they appear.

The debate whether post-primary TB is actually a re-infection or reactivation of an old lesion is a source of much controversy. Lucas (1994) estimates that 5-10% of all TB diagnosed in childhood will reactivate at some later stage in the infected individual's life, often aided by the onset of old age, diabetes, or alcohol or drug abuse. Studies by pathologists during 1927, found that one quarter to one half of patients with radiologically normal lungs revealed at autopsy, old calcified tuberculoid lesions in the lung, suggesting that an effective protective immunity - consisting of a complex interplay between infected and bystander macrophages, as well as the interactions of T-lymphocytes, both helper and cytotoxic - is responsible for the low incidence of disease progression.

One of the most characteristic differences between re-infection and reactivation is the position of the infective focus in the lung. As discussed earlier, a primary TB infection usually initiates in the mid-lower zone of the lung, as the bacilli are breathed in from the outside, whereas reactivation disease is frequently in the upper zone of the lungs, suggesting dissemination of the original infection. These lesions are also necrotic in nature, suggesting a prior sensitisation to the tuberculin toxin, and subsequently on reactivation a hypersensitivity to the tuberculin protein, resulting in extensive necrosis.

Due to the varied disease patterns, it has been suggested by Lucas (1994) that in the immune-competent individual, a classical tuberculoid granuloma is evident, with epithelia cells and giant cells in the granuloma, but little or no accompanying necrosis or caseation, and subsequently few culturable AFB. In the immunocompromised host,

a different granulomatous reaction is described, classified as the non-reactive immune response, characterised by granuloma necrosis with extracellular AFB in large numbers, with few giant and epitheloid cells. A range of varying granulomas are seen amongst different individuals and with various strains of bacilli. Interestingly, this non-reactive granuloma is not seen in *M. avium, M. leprae* or *M. intracellulare* infections, as these bacilli do not seem to have an adverse effect on the macrophages, apparently allowing symbiotic growth within the macrophage. In contrast, *M. tb.* infection appears to have a toxic effect on macrophages, resulting in cell lysis with large numbers of extracellular bacteria. In HIV, the immunocompromised host has a 10% annual risk of developing TB, as compared to the 10% lifetime risk of the immunocompetent individual. This together with the high level of mortality, often 90% of cases within 4-16 weeks, and the high incidence of MDR TB contracted by HIV sufferers, has significantly contributed to the resurgence of TB in the Western world. The rapid initiation and progression of the disease in these individuals is thought to result from the inability to orchestrate delayed-type hypersensitivity required to activate macrophages and start the CMI. The inability to evoke this response is directly related to the destruction of the T-helper lymphocytes by the HIV virus, explaining why the progression of TB in HIV is so rapid.

It remains perplexing that, apart from immunocompromised individuals, only 5-10% of healthy people experience a breakdown in their protective immunity, allowing establishment of clinical disease. The reasons for this are still widely researched and may be related to the genetic makeup of an individual or the bacilli, as discussed further in Chapters 3 and 4.

1.7 In Conclusion:

The history of tuberculosis is ancient, and yet the disease remains one of the greatest scourges of the developing world. As new and more lethal diseases emerge, tuberculosis can only continue to increase, as it is one of the most opportunistic and virulent facultative intracellular pathogens known to man. The fact that the bacteria can apparently survive within the host for decades, only to emerge when the individual is immuno-compromised, suggests that the pathogen cannot easily be eradicated at a cellular level. Unfortunately, *M. tb.*, although known for over one century, is poorly understood, and much of the bacteria's ability to invade and colonise the host is still under intensive investigation. The molecular details of the interaction between the inhaled bacilli and the host phagocyte, the macrophage, remain mysterious. It is well documented that *M. tb.* enters host macrophages and seems to be able to orchestrate the entry to its advantage, but the mechanisms are debatable. In approaching this problem, it is instructive to consider the strategies used by pathogens. On this basis, one of the most likely portals of entry to be exploited by *M. tb.* is the cell-surface complement receptor type 3, (CR3), also known as Mac-1, Mo-1, or CD11b/CD18. As described in ensuing chapters, CR3 is required for many cell-cell and cell-matrix interactions and is a key receptor in the migratory abilities of the immune cells. To target this receptor and to exploit it to the bacteria's advantage would require either a bacterial surface moiety that can provide a CR3 ligand or the ability to bind opsonins (mainly complement components) that are known to interact with CR3. To explore this, we have designed a research strategy to aid in demonstrating the requirement for CR3 and the conditions needed for the bacteria to interact with this receptor.

CHAPTER TWO

STATEMENT OF HYPOTHESIS, AIMS, AND STUDY SIGNIFICANCE

2.1 HYPOTHESIS:

Mycobacterium tuberculosis is a well known facultative intracellular pathogen that multiplies both intracellularly and extracellularly in its host, the human. The ability of the pathogen to invade and survive within its host cell, the mononuclear phagocyte, is a critical component of its virulence modus operandi, in that it provides it with a distinct advantage over host defences. **We hypothesize that the bacterium evades normal host defence mechanisms by binding directly (ie, nonopsonically) to complement receptor type 3 (CR3), by means of a specific, CR3-binding cell surface ligand. CR3 is a key macrophage receptor and adhesion protein, and is a frequent target of intracellular pathogens because CR3-mediated phagocytosis is not accompanied by a respiratory burst. Nonopsonic binding to CR3 may be critical in the complement-poor alveolar space, and, we speculate, may further enhance intracellular survival by unknown mechanisms.**

2.2 AIMS:

The central aim of this thesis is to study the interaction between CR3 and *M. tb.*, with particular reference to the role of serum opsonins.

This will be accomplished by:

1. Examining the capacity of human peripheral blood monocyte-derived macrophages to phagocytose *M. tb.* in the absence of complement.
2. Heterologously expressing human CR3 in a non-phagocytic cell line to investigate the direct interaction between *M. tb.* and CR3 without the involvement of other macrophage surface receptors.
3. Determining the extent to which serum opsonins are required for the interaction between *M. tb.* and CR3-expressing cells.
4. Establishing whether the C3bi-binding site of CR3 is involved in the interaction with *M. tb.* in the absence of complement.

2.3 STUDY SIGNIFICANCE:

The ability to invade, survive and establish longterm persistence in host mononuclear phagocytes is the single most pathogenic feature of *M. tb.* For reasons to be explored in detail in this thesis, complement receptor type 3 (CR3) is hypothesised to be the preferred receptor for *M. tb.* Moreover effective use of this receptor by *M. tb.* in the

establishment of the initial infection in the complement-poor, quiescent lung likely depends on direct, nonopsonic binding by the bacilli. It is the aim of this study to rigorously establish whether direct, nonopsonic binding of *M. tb.* to CR3 can occur. If so, it is strong evidence for the existence of a specific, CR3-binding ligand on the surface of the pathogen. The isolation and identification of such a ligand will be the subject of later work, and it offers the prospect of designing novel preventive and therapeutic strategies directed at blocking the critical step of pathogen-host cell binding.

CHAPTER THREE

MYCOBACTERIUM TUBERCULOSIS CULTURES

3.1 INTRODUCTION:

Mycobacteria are a large group of Gram-positive bacteria that cause a wide range of diseases in humans and animals, which may be localised or disseminated in nature. Most Mycobacterial species are saprophytes that inhabit water or soil and it is likely that modern pathogens evolved from saprophytic ancestors, adapting to infect their current hosts. Tuberculosis-like infections contracted by humans in earlier millennia were thought to be of *M. bovis* origin, which probably evolved to establish the highly infectious human pathogen, *M. tb.* (Bloom, 1994) (see also Chapter 1).

Mycobacteria are classified into four broad categories, according to their natural habitat, degree of pathogenicity, and hosts. These categories are a) obligate pathogens, exclusively found in humans; b) facultative pathogens, primarily found in animals or the environment, but can cause human infections; c) opportunistic, environmental pathogens, that can take advantage of the immune compromised human; d) saprophytic bacteria, mainly environmental, with no pathogenicity in humans. Table 3.1 displays the vast number of mycobacteria identified and which are classified according to the above criteria (Berlin, 1990).

Mycobacterium tuberculosis (*M.tb*) is a facultatively intracellular, obligate pathogen, which is striking by its ability to invade and persist in human mononuclear phagocytes.

Table 3.1: Recognized Species of the Genus Mycobacterium

(Adapted from Berlin, 1990)

	INTRACELLULAR PATHOGENS		OPPORTUNISTIC PATHOGENS	SAPROPHYTES
	OBLIGATORY	**FACULTATIVE**		
SLOW GROWERS				
STRICT PATHOGENS	*M. africanum* *M. leprae* *M. ulcerans*	*M. bovis* *M. tuberculosis* *M. asiaticum* *M. kansasii* *M. marinum* *M. simiae* *M. scrofulaceum* *M. szulgai* *M. xenopi* *M. avium* *M. haemophilum* *M. intracellulare* *M. malmonense* *M. shimoidei*	*M. gordonae* *M. flavescens* *M. gastri* *M. nonchromogenicum* *M. terrae* *M. triviale*	

ANIMAL PATHOGENS	*M. farcinogens* *M. lepraemurium* *M. porcinium*	*M. microti* *M. paratuberculosis* *M. senegalense*		
RAPID GROWERS		*M. chelonae* *M. fortuitum*	*M. fallax* *M. smegmatis*	*M. agri* *M. aichiense* *M. aurum* *M. chitae* *M. chubuense* *M. duvalii* *M. gadium* *M. gilvum* *M. komossens* *M. neoaurum* *M. obuense* *M. phlei* *M. pulveris* *M. Rhodesiae* *M. sphagni* *M. tokaiense* *M. vaccae*

It is a slow-growing mycobacterium that is spread from one host to another by means of small aerosols of 1-5μm in diameter, expectorated from the lungs of an individual with active pulmonary disease. These droplets are able to pass unnoticed through the bronchial passages into the alveoli of the lung, where the bacilli adhere to and invade the alveolar macrophages. As described in Chapter One, a wide range of pathological scenarios may ensue from the initial infection, all dependent on the host's ability to evoke an immune response and the bacilli's ability to withstand the attack. As the disease progresses, the bacilli may be forced to revert to their earlier saprophytic nature and feed off the necrotic matter encasing them in the centre of necrotic lesions (Lucas, 1988; Lucas, 1989).

Mycobacterium bovis is a slow-growing pathogen which was originally a soil bacterium. Its natural host is cattle but it may cause disease in humans through oral ingestion of contaminated milk. The disease is usually limited to the gastrointestinal tract and lymph nodes, but has a similar pathogenesis as *M.tb*. The incidence of *M. bovis* infections in humans has declined since the introduction of pasteurisation procedures, with destruction of the bacilli in dairy products, but may still occur in rural or developing communities.

Bacille Calmette-Guerin (BCG) is a laboratory strain of mycobacteria not found in the wild, and the origin of which is somewhat disputed, but which is thought to be a unique strain of *M.bovis*. It has been used extensively as a vaccine to protect infants against *M.tb*. BCG disease in humans is rare and usually due to inability to cope with the subcutaneous load used in inoculations (Lucas, 1989).

Mycobacterium leprae is an extremely slow growing, obligate intracellular pathogen whose only natural host is humans, and which can not be cultured *in vitro*. In many

respects, it is an extreme representation of the pathogenic mycobacteria, highlighting the many problems encountered with mycobacteria *in vitro*. *M. leprae* infection is fascinating, as it passes through three stages in the host and only at the last stage is the host able to recognise and combat the infection. The initial stage occurs in the skin of the host, where the bacteria replicate undisturbed, until they disseminate to peripheral nerve endings and establish the second stage of infection. Once the final tuberculoid, granulomatous stage is reached, the host attempts to mount a cell-mediated immune (CMI) response to eradicate the infection, which by now is often rampant (Berlin, 1990; Bloom, 1994). One of the most striking differences between *M. tb.* and *M. leprae* infection is the absence of necrotic matter in leprosy, due to its silent intracellular growth, whereas *M. tb.* is a much more toxic intracellular pathogen.

The rate of infection and the severity of the disease, as well as the ability of the host to respond, depend on the virulence of the bacterial strain and species, which are not fully understood. It was thought that the slower growing pathogens, such as *M.tb, M.leprae* and *M. avium*, are more virulent than the fast growers, such as *M. smegmatis* and *M. fortuitum*, but this is not entirely true, as the laboratory strain H37Rv, a virulent strain of *M.tb*, is faster growing in the guinea pig, due to its virulence, than the less infectious, avirulent strain, H37Ra, which is also a slow grower *in vitro*, suggesting that the growth rate *in vitro* has little significance to the virulence of the pathogen *in vivo*. The search for virulence determinants and factors is underway in laboratories throughout the world.

The natural characteristics of these bacteria make them difficult to culture *in vitro*, if at all, and pose a number of basic fundamental obstacles that will be discussed under Materials and Methods in this chapter. Understanding the complexity of the bacterial

cell wall and the growth characteristics of the bacteria may provide insight into a possible ligand on the cell wall that could interact with host cell receptors such as CR3. Similarly, understanding different growth stages, some of which may be more invasive to host cells, would also aid in the infection assays.

3.1.1 Cell Wall Structure:

The characteristic cell wall, which has earned the Mycobacterium species its identifying hallmark, namely the ability to retain dye in its cell wall after acid washing, commonly referred to as acid fastness, is often said to be responsible for the bacteria's pathogenicity and resistance against eradication. However the cell wall, although previously thought to be unique, maintains close similarities with that of other Gram-positive bacteria, such as *Nocardia, Corynebacterium;* and *Rhodococcus.*

Remarkably, the cell wall consists of greater than 60% lipid. The cell wall core comprises of a complex, covalently linked, tripartite polymer, consisting of a peptidoglycan backbone, an arabinogalactan layer, and an outerlayer of waxy mycolic acids. Each of these are covalently linked to each other to provide a semi-rigid supramolecular structure. The peptidoglycan skeleton is similar to those seen in Gram-positive bacteria, made up of polysaccharide chains of glucosamines and muramic acids that are cross-linked to tetrapeptide side chains. The arabinogalactan layer is covalently linked to the peptidoglycan muramic acids, by means of galactose homopolysaccharides, similar to those identified in *Corynebacterium* and *Nocardia.* The mycolic acids are esters that terminate in arabinose residues, with high molecular weight ß-hydroxy fatty acids and long α side chains. These structures are similar to

waxes in nature, as identified in the above mentioned species. A number of other glycolipids, glycopeptidolipids, and proteins are non-covalently associated with these structures, and perform different functions for the bacteria. Of these an important polysaccharide, lipoarabinomannan, LAM, with arabinose and mannose subunits covalently linked to a phosphatidylinositol tail, provides immunological activities similar to lipopolysaccharide, LPS, well described in Gram negative bacteria. Trehalose-based glycolipids provide the characteristic "cord factor" that is often said to be indicative of bacterial virulence, including sulphated acyl trehaloses that form the basis of the surface sulfatides found on mycobacteria. Finally, there are a host of phenolic glycolipids, peptidoglycolipids, and ceramides (true waxes) that interdigitate with the mycolic acids and contribute to the well-described lipophilicity and waxiness of the outer envelope (Ehlers, 1994; Douglas and Duncan, 1995).

3.1.2 Metabolism and Nutrient Requirements:

Mycobacteria have a range of growth rates that seem to broadly, but not entirely, indicate the pathogenicity of the bacterial strain, with the slower growers being the most pathogenic and the faster growers the least pathogenic. The slow growers are greatly retarded when compared to the replication rate of 20 minutes of *E. coli*, as they take on average 12-20 hours to divide, whereas the faster growers divide approximately every 1-2 hours; in all cases, division is by means of binary fission. A possible reason for the slow growth may be the rigidity of the cell wall and the interesting paucity of rRNA genes (1 copy versus 7 in *E. coli*). The lack of a requirement for essential nutrients and the ability to exist in relatively oxygen-depleted

environments enable these bacteria to exist in harsh conditions and compete in nutrient-poor environments for basic salts and carbon sources.

These bacteria have adapted remarkably to the famine-or-feast cycle characteristic of natural environments, including the immunocompetent host, and seem to have won the survival race against the human. Unfortunately, simulating *in vivo* conditions during *in vitro* culture is extremely difficult, and much has still to be optimised and understood before we can rid ourselves of the animal model (Davis et al., 1967; Wayne, 1994b) .

3.1.3 Growth Characteristics of *Mycobacterium tuberculosis:*

The type of culture media and the conditions of growth are fundamental in dictating the physiology, pathogenicity, and antigenicity of the organism, and should be adapted to suit the eventual aim of the experiment.

For a number of years the bacteria were thought to survive only in rich media containing complex nitrogen, lipid, and carbohydrate components, but subsequently this has been found to be relevant only in the subculture of clinical isolates, where accompanying tissue destruction probably inhibits the growth of the bacteria and therefore the coaxing of bacterial growth is required. For experimental purposes, a simple salt solution with the addition of a nitrogen and carbon source are the only nutrients essential for growth. The oxygen provision will affect the growth rate and virulence state of the bacteria. A number of articles have been published to validate the requirement of, for example, glycerol or a detergent to enable effective *in vitro* culture of the bacteria, but this complicates the growth pattern of the bacteria, as the ultrastructural alterations and accompanying effects are not physiological. It is of great

importance to remember that no *in vitro* culture will be homogeneous, but rather a heterogeneous mixture of bacilli at different growth stages and ages, and with different virulence characteristics (Wayne, 1994b).

The temperatures at which the bacteria are reported to grow range quite broadly, from between 29°C to 39°C, with the optimum being around 37°C, and this affects the growth rate of the bacteria significantly. Similarly, the controversy of stationary versus shaking cultures depends on the type of bacterial culture required. Stationary bacteria will grow as a pellicle that thickens with age and may contain large amounts of cord factor, whereas shaking bacteria, depending on the type of shaking, will grow in small clumps of suspended bacteria. The dispersion of these clumps and the number of bacilli per clump affects the inoculum given to animals *in vivo* and to cells *in vitro*, as the particulate size may dictate the fate of the bacilli and the response of the cells or tissues (Wayne, 1994a).

Reported growing techniques suggest a requirement for additional quantities of either glycerol or Tween 80, a detergent, as aids in providing a relatively single-cell suspension. These factors were considered, but decided against in our culture protocols, as their effects, when in large volumes, on the bacteria were potentially too non-physiological for the type of experiments planned. High percentages of glycerol not only may alter the normal metabolism of the bacteria, but may also result in excessive production of lipids and polysaccharides. Similarly Tween 80 may be toxic to the bacteria, due to its high oleic acid content. The oleic acid can be mopped up by the presence of albumin, but this again may alter the extrinsic surfaces of the bacteria and mask secreted antigens, thereby detracting or adding to the pathogenicity of the bacteria. It was therefore at the risk of not being able to generate single-cell

suspensions, that the bacteria were cultured in the most physiological conditions possible, to minimise the introduction of culture artefacts (Berlin, 1990; Wayne, 1994b).

Due to the nature of mycobacterial growth, it is difficult to obtain a single culture in which all the bacteria are synchronised, with respect to their growth stage. This has been said to be due to the characteristic clumping or pellicle growth observed during culturing. The bacteria are thus in differing degrees of contact with nutrients and oxygen, and therefore tend to be at various stages of the growth curve. Recently it has been suggested that if a bacterial culture is allowed to reach stationary phase and maintained there for some time, all the bacteria will shut down DNA synthesis and become "dormant", but not dead. Such bacteria, when inoculated into fresh media and exposed to more oxygen, will then all be synchronised to start RNA synthesis and divide within 8 hours, whereafter they will begin to synthesise DNA, suggesting that these cultures will be synchronised for 24 hours after re-inoculation (Wayne, 1994a).

3.2 MATERIALS AND METHODS:

3.2.1 Culture Media:

Bacteria were cultured initially on Loewenstein Jensen (L-J) agar slopes (Addendum A.2) to establish an identifiable culture as indicated in Figure 3.1. The bacteria were then grown in liquid Kirchner's medium (Addendum A.2), which consists of a low quantity of a simple carbon source, of 2% glycerol, and Asparagine as the nitrogen

source, together with a mixture of essential minerals and salts. The pH of the medium was maintained at 7.2. The medium is bulk autoclaved at 15 lb./sq. inch for 20 minutes. No detergents were added to the culture.

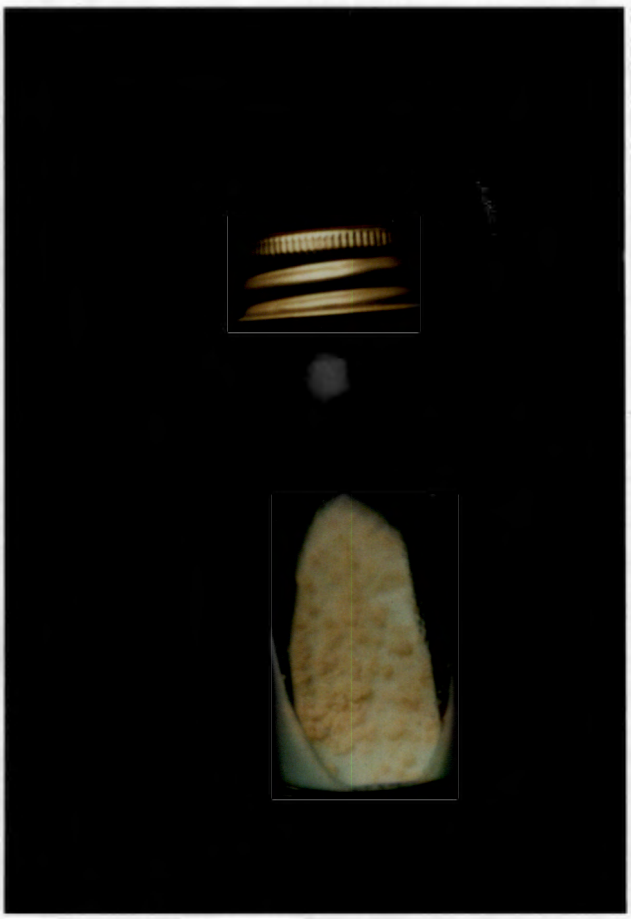
3.2.2 Bacterial Stocks:

Stocks of *M. tb.*, strain H37Rv 27294, were obtained from the ATCC as lyophilised cultures and reconstituted on L-J slopes at 37°C for 2-3 weeks, before being cultured for experimental use.

3.2.3 Culture techniques of *M. tb.*:

The original ATCC stock grown on L-J slopes was washed in 3 ml Kirchner's medium and gently dislodged with a sterile cotton bud. The dislodged bacterial suspension was aspirated and transferred into a sterile tube. The suspension was then forced through the tip of a 1-ml Gilson pipette, to break up the largest clumps. Once an approximately homogeneous suspension was achieved, a further 5 ml Kirchner's was added to the suspension and mixed well. The suspension was left at room temperature for 10 minutes to precipitate out the largest clumps and the top 5 ml of generally single-cell bacteria was carefully removed and added to 100 ml of fresh Kirchner's medium in a 500-ml glass bottle. The liquid culture was then incubated, shaking at 200 rpm, at 37°C for two weeks, before inoculating the final bacterial stock culture. (The 100 ml culture was grown first to provide a higher density inoculum for the final stock culture). Fifty millilitres of the two-week-old culture were inoculated into 200 ml of fresh Kirchner's medium in a 1-litre glass bottle and incubated under the same

Figure 3.1: Loewenstein-Jensen slope of *Mycobacterium tuberculosis* (H37Rv)



Both clinical and laboratory samples of *M. tb.* are streaked onto LJ slopes to aid selection from co-contaminants.

conditions for 7 to 10 days. At this stage the bacteria comprised an opaque culture with a few uniform clumps in suspension. The culture was aliquoted into 0.5 ml aliquots, and frozen at -20°C for subsequent use in experiments. Quantitation and screening for contamination were done at each step as described below.

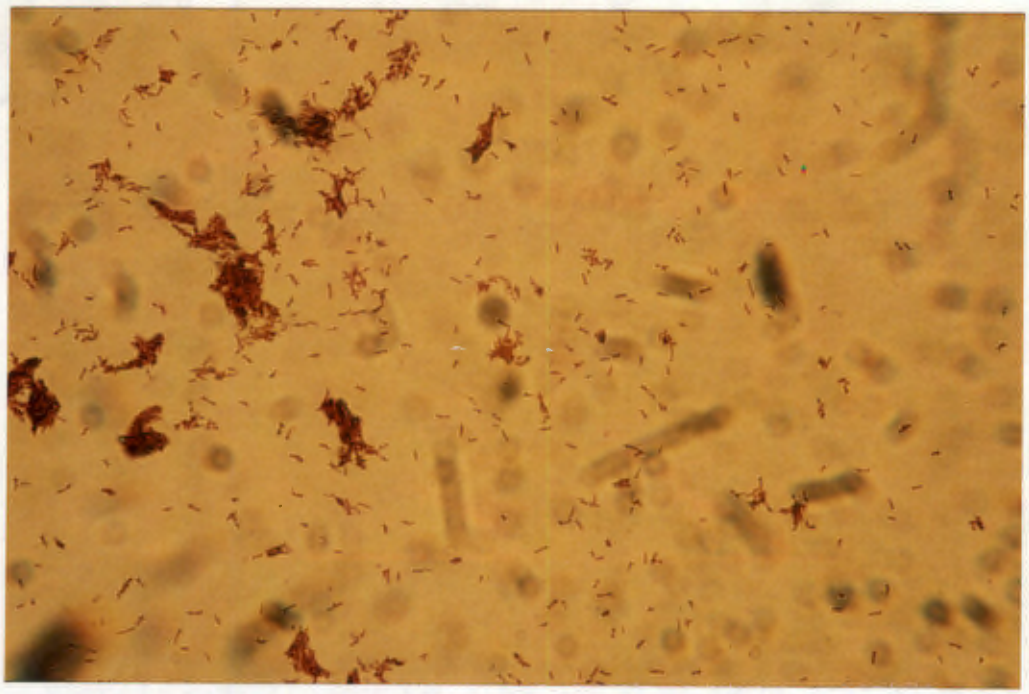
To ensure that the mycobacterial stock cultures were not contaminated with adventitious micro-organisms, regular Ziehl-Neelsen (ZN) staining of aliquots of the cultures was performed (Addendum A.1.15). The ZN smears gave an indication of the state of the bacteria and revealed any cross contamination that may have occurred. All contaminated cultures were discarded after autoclaving.

3.2.4 Quantitation of Bacterial Stocks:

Although this is not accurate, bacterial cultures were quantitated by visual turbidity, by comparison to McFarland's standards (Addendum A.2) that are used for quantitating *E.coli*, and also by means of optical density at 550 nm. To validate these readings, an aliquot of the suspension was plated for colony forming units (CFU) on Kirchner's agar and incubated for three weeks at 37°C with 5% CO_2.

Before quantitation, all bacterial aliquots were first syringed through various gauges of needles culminating in an insulin syringe (Addendum A.1.16). This aided in providing a relatively single-cell suspension, as viewed by light microscopy. As indicated in Addendum A.1.17, most bacterial cultures were grown to approximately McFarland's standard 3 or 4, with an optical density of 0.408 at OD 550. The CFU were not that accurate in determining the number of bacteria present, as the culture method produced small clumps of bacteria even after sequential syringing to disrupt the

Figure 3.2: Ziehl-Neelsen stain of *Mycobacterium tuberculosis* (H37Rv)



To ensure no co-contamination of the H37Rv culture has occurred, bacteria are heat-fixed onto glass slides and stained with carbolfushcin, followed by acid washing to differentiate between acid-fast bacilli and contaminating Gram positive bacteria.

clumps, and this results in an underestimate of the true number of bacteria where this is based on CFUs.

Viability of all cultures was established qualitatively at each experiment, by culturing an aliquot on both L-J slopes and in Kirchner's medium.

3.3 RESULTS OF BACTERIAL CULTURES:

H37Rv was grown and stored as described above. The bacteria were examined microscopically after ZN staining and displayed features of characteristic *M. tb.*, as shown in Figure 3.2. The rod-like bacilli were either in small clumps of 3-5 bacteria or as single pink rods across the slide. No blue Gram-negative contaminants were present.

The bacterial culture that was used for most of the experimental assays was checked for viability by reculturing on L-J slopes, which indicated growth after 2 weeks at 37°C.

The bacterial stock culture was quantitated by comparison to McFarland's standard 3 or 4 and resulted in an approximate value of 12×10^8 organisms per ml of culture medium. Similarly, an aliquot of the culture diluted 1:2 in Kirchner's media gave a reading of 0.408 at OD 550, which is equivalent to 6×10^8 organisms per ml (Smibert and Kreig, 1994).

All the bacterial aliquots were stored at -20°C in the culture supernatant until required, and no culture was defrosted and then refrozen.

3.4 CONCLUSION AND DISCUSSION:

During the course of this study, additional literature became available about the effects of various culture methods and storage techniques on *M. tb.*, and gave rise to a number of interesting questions regarding our method of culture. First, shaking the bacteria might be the most "unphysiological" method of bacterial culture, whereas a slow-pulse shake may provide the necessary agitation to prevent cord formation, but not result in the solid small clumps encountered with continuos shaking, and therefore mimic growth in the alveoli more closely. What effects our culture method has on the bacterial physiology is unknown, but it is certainly worth investigating further. Second, attempts at synchronising the bacteria are also important in the infection assays, as it would be of interest to identify at which stage the bacteria express the relevant cell wall moiety that interacts with the complement receptor type 3 (CR3) or other host cell receptors.

Culture techniques similar to ours were used by both Schlesinger et al. (1990b) and Stokes et al. (1993) for the preparation of their bacterial inoculum. Schlesinger cultured his bacteria on Midddlebrook's 7H11 agar for 9 days at 37°C with 5% CO_2, after which he scraped off the bacteria and resuspended them in RPMI with 20 mM HEPES and 3 glass beads and vortexed the suspension to disrupt the clumps. The suspension was left for 30 minutes at room temperature, before aspirating the top half of the suspension, which contained single-cell bacteria and these were used in his infection studies. Similarly, Stokes grew his Erdman to late log phase in Proskauer and Beck medium with 0.05% Tween 80, which he then aliquoted and froze at -70°C

until required. Before inoculation, the bacteria were resuspended by syringing through a 25-gauge needle, to break up clumps.

In an attempt to standardise the growth stages of our cultures, a comparison was made to a bacterium similar in nature and growth characteristics to mycobacteria, namely *Nocardia.* These organisms, although dividing at a faster rate than *M. tb.*, establish a similar infection and mode of host entry, and have been reported to cause confusion in the diagnosis of tuberculosis in areas of cross-contamination. Although my growth stages were based on *Nocardia* growth curves, the true growth rates for *M.tb.* are still unknown. Once these are identified and the bacteria can be cultured in a synchronised fashion, the importance of true log-phase or stationary-phase cultures on infectivity can be assessed. *M. tb.* has approximately a 3.33-fold longer division cycle than Nocardia, making the log phase anywhere from 3 to 10 days after initial inoculation. Usually at this stage the bacteria are too sparse in number to provide an efficient inoculum, and it was therefore decided to prime the final culture with a two-week growth that contains, it was hoped, bacteria at or near early stationary phase; the final culture was then grown for 7 to 10 days.

A third question concerning the method of culture was the rate and level of oxygenation that is required for both optimal and "physiological" growth. This is relevant to the *in vivo* setting in which the bacteria apparently survive in microaerophilic or even anaerobic conditions in the necrotic lung. Such conditions may alter the virulence and pathogenicity of the bacteria, in particular in regard to our interest of invasion of host cells.

Despite the validity of these questions we have generated bacteria under defined conditions, similar to others, and have consistently used these stocks in our infection

experiments. Further comprehensive analyses of bacterial variations in growth stages and environmentally determined expression of virulence factors will be required to gain a fuller understanding of the *M. tb.*-host cell interaction. However, for the purposes of this study, the defined bacterial stocks generated have been adequate in performing the experiments described in the ensuing chapters.

CHAPTER FOUR

MACROPHAGE - MYCOBACTERIAL INTERACTIONS

4.1 INTRODUCTION:

4.1.1 The origin and kinetics of macrophages:

Macrophages are the most primitive but versatile immune cells of the body. Not only do they aid in normal maintenance and repair functions, but they are also the main defence cells encountered by invading pathogens. Macrophages are the major differentiated cells of the mononuclear phagocyte system and are distributed widely throughout the body. Their structural and functional heterogeneity allows them to participate in a variety of physiological and pathological processes, although they all originate from the colony forming unit, granulocyte-macrophage, (CFU-GM), in the bone marrow. Interestingly, the same progenitor gives rise to the neutrophil (Auger and Ross, 1992; Stewart et al., 1994).

Macrophage precursors - monoblasts, promonocytes and monocytes - differentiate within the bone marrow before migrating into the blood stream as monocytes. These precursors of the macrophage differ in morphology and ultrastructure from the macrophage as described in Figures 4.1 and 4.2, depicting the characteristics of each cell stage.

The kinetics of these cell stages differ from species to species. In the mouse, the monocyte remains in the bone marrow for less than 24 hours before emigrating into the

Figure 4.1: The development of macrophages and their precursors

(Source: Lewis C.E., and J.O'D. McGee. The Macrophage. 1992. IRL Press)

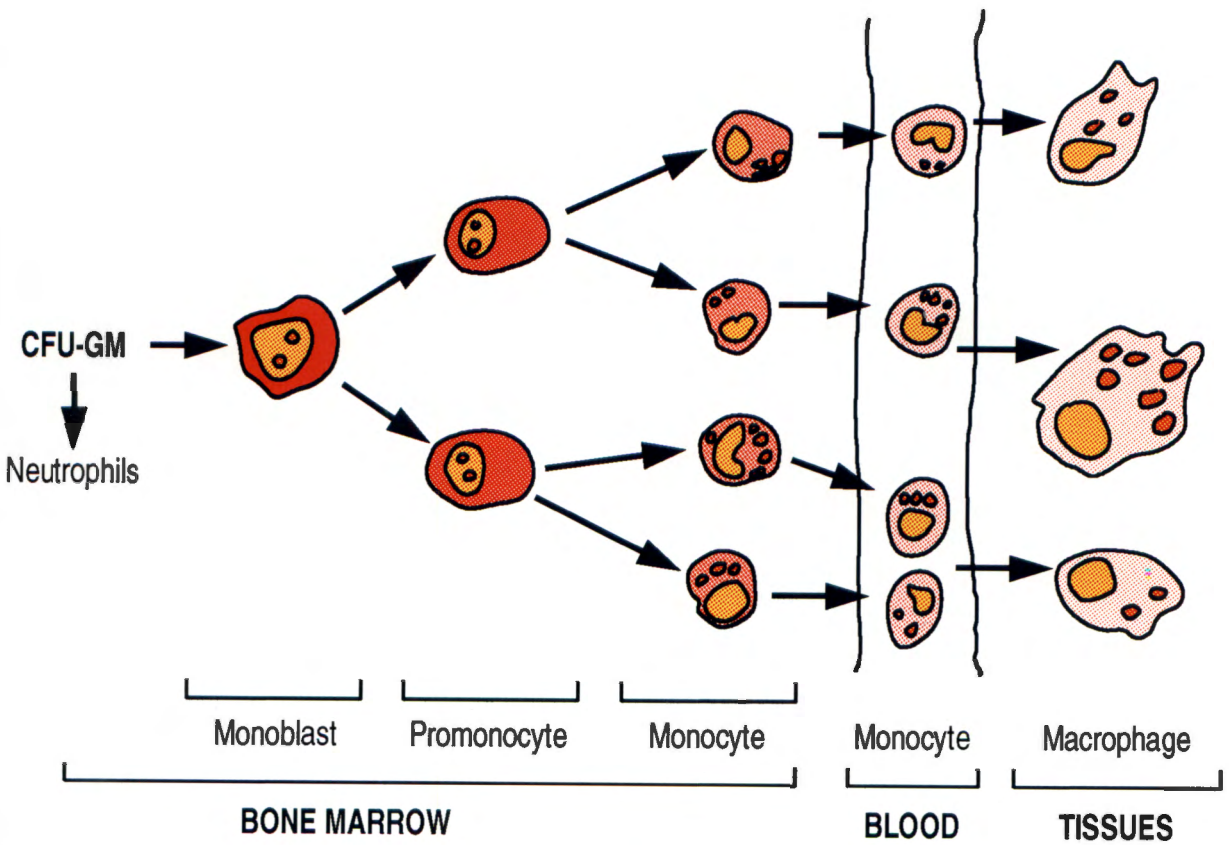
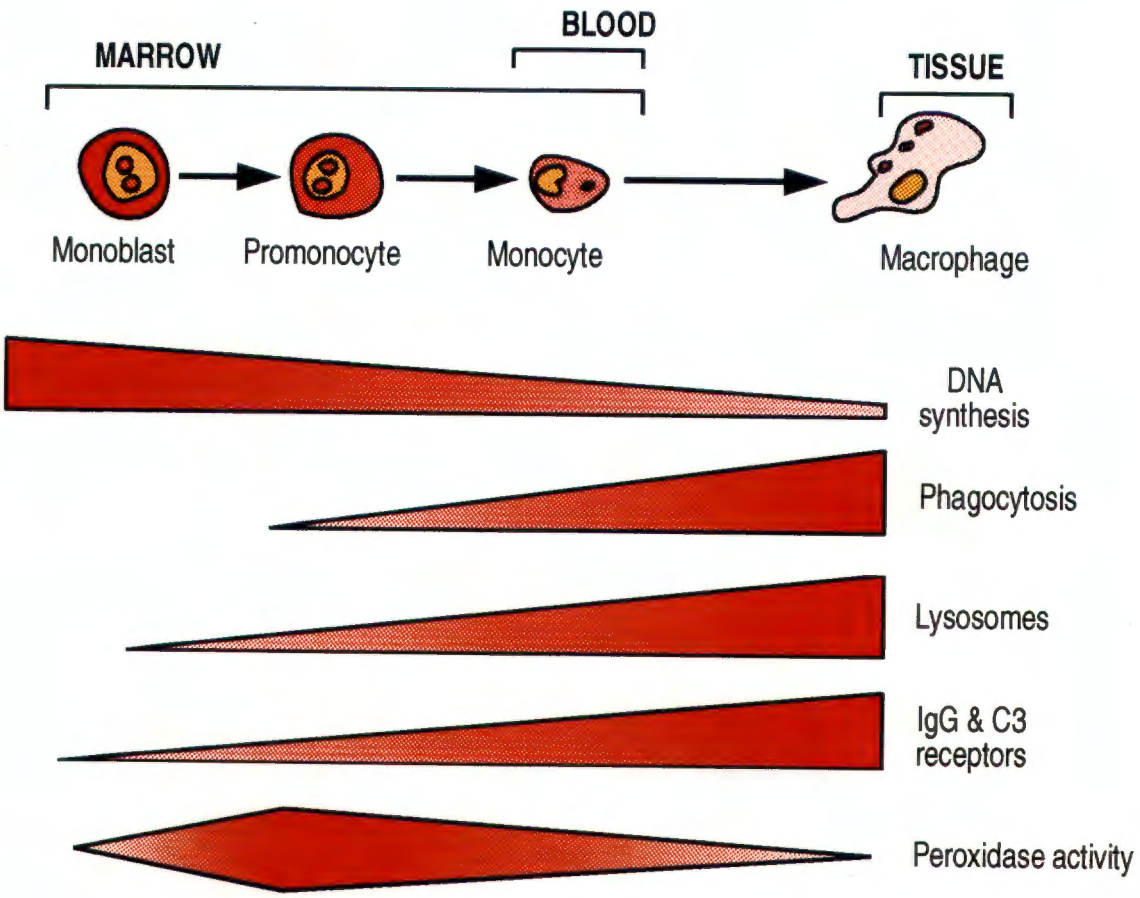


Figure 4.2: Characteristics of developing macrophages

(Source: Lewis C.E., and J.O'D. McGee. The Macrophage. 1992. IRL Press)



blood stream where it will circulate for 25 hours before leaving the blood stream for its final destination in the tissues. In contrast, in man the monocyte remains 70 hours in the circulation before egress into the tissues. The process of tissue selection appears to be a random event, unless there is an inflammatory response to which the cells will migrate en masse (Auger and Ross, 1992; Stewart et al., 1994).

Most macrophages found in organs have migrated there from the blood stream as monocytes and will finally differentiate into macrophages upon entering the organ. At this stage, the macrophage does not recirculate into the blood stream, but remains in the tissues for several months before either dying there or migrating to lymph nodes to senesce. Recently it has been found that 5% of macrophages found in the tissues are the result of the division of mononuclear phagocytes arriving directly from the bone marrow as promonocytes and then completing their division *in situ*. A small fraction of bone marrow-derived monocytes will alter morphologically, in response to a particular anatomical position and accompanying environmental stimuli, derived from the extracellular matrices and local cell signalling, to represent a group of highly specialised macrophages classified as resident macrophages. These cells are found in specific sites in the normal tissue and may participate in inflammation, but generally perform specialised functions in the organ in which they reside, such as the specialised scavenging functions of Kupffer cells in the liver and macrophages in the spleen (Crocker and Milon, 1992; Auger and Ross, 1992; Stewart et al., 1994).

4.1.2 The morphology and distribution of the macrophage:

The monocyte differs from the macrophage in both size and ultrastructure. The monocyte is a small rounded cell, approximately 12-18 μm in diameter, with a large

irregular nucleus, whereas the macrophage is much larger, 25-50 μm in diameter, and varying in size and shape, with a ruffled surface and many large intracellular structures.

Macrophages adapt to the organ or tissues in which they mature, often leading to heterogeneity amongst resident macrophages. They may function with antibacterial activity in the mucosal wall of the gut or as a filter in the lymph. Alveolar macrophages are thought to reside in the lung for approximately three months before migrating to lymph nodes to die. During their residence in the lung they are the first line of defence against invading pathogens or foreign particles, and only after being overwhelmed will they recruit circulating monocytes and neutrophils. Alveolar macrophages adapt to deal with these events by altering their ultrastructure to contain large membrane-bound vesicles containing proteolytic enzymes. They also play important roles in the formation of granulomas and walling off of persistent pathogens (Auger and Ross, 1992; Adams and Hamilton, 1992).

Other protective sites in which macrophages find themselves are the liver, spleen, intestine and artery walls. Giant cells are thought to be the terminal stages of the macrophage, as they comprise a multinucleated cell with large intracellular vacuoles containing a variety of particulate matter. These cells, although they have lost the ability to phagocytose as effectively as macrophages, contain large stores of lysosomal and respiratory enzymes, suggesting a functional role in the inhibition of intracellular pathogens. Therefore, not unexpected, they are a major cell type found in granulomas (Adams and Hamilton, 1992; Stewart et al., 1994).

4.1.3 Macrophage cell-surface receptors:

To provide effective communication between itself and the environment, the macrophage displays a vast number of cell surface receptors that not only keep it informed of the external milieu, but also enable it to differentiate, migrate, and respond to extracellular stimuli. Many of these receptors have been found as a result of interactions with ligands or after the generation of panels of cell surface-specific MAbs, and many more are still to be explored. Often these receptors will not work in isolation, but rather as a stimulus to initiate a cascade of intra- and inter-cellular events (Celada and Nathan, 1994; Mosser, 1994; Crocker and Milon, 1992).

The receptors of greatest interest to us are those that induce the macrophage to phagocytose foreign particles. Of these, the Fc receptor and complement receptors are the most frequently discussed. The Fc receptor binds to the Fc region of the Ig molecules, which in turn stimulates intracellular signalling events that initiate cytoskeletal rearrangements and promote phagocytosis of the Ig-coated particle. Similarly, the complement receptors CR1, CR3 and CR4 interact with the C3b and C3bi fragments of the complement cascade to promote phagocytosis of complement-coated, opsonised particles (Mosser, 1994; Speert, 1992).

The receptor -ligand interaction is often dependent on the nature of the bacterial surface, but once the bacteria has been opsonised by complement components, IgG and fibronectin, the bacteria interact with these specified receptors, such as the complement receptors, FcR, and fibronectin receptors, which in turn promote their phagocytosis (Silverstein et al., 1989; Horwitz, 1982). Depending on the phenotype of the macrophage, its receptor expression will also be limited as demonstrated by the

lack of CR3 on Kupffer cells as opposed to the high expression levels seen in circulating monocytes (Gordon et al., 1988). Similarly FcχR differs depending on the tissue distribution of the cells, as shown by high levels of FcχRIII in neutrophils and macrophages, as compared to circulating monocytes (Unkeless, 1989). It is therefore not suprising that the melieu in which the macrophage resides may often dictate its receptor expression, as seen in macrophages stimulated by IFN-gamma during an immune response, where the levels of surface FcχR are enhanced to interact with any opsonised particles (Wright et al., 1986). Similarly, macrophages that are resident in tissues that are more frequently in contact with foreign particles, will display a vast number of surface receptors that aid phagocytosis, but this increase in receptor expression is not all that is required for effective phagocytosis as the activation state of the cells is crucial to the fate of the particle. A number of experiments performed using C3bi-coated erythrocytes clearly indicated this requirement, by showing that these coated cells were able to bind effectively but not induce phagocytosis, which suggests that a more active communication is required, as indicated by the process of "zippering" phagocytosis discussed in a later chapter (Griffin et al., 1975; Wright and Silverstein, 1982).

For bacteria that appear to be non-opsonised the macrophage has receptors or receptor epitopes that are able to bind to moieties either directly on the bacterial cell wall, or by utilising serum opsonin such as mannose binding protein to interact between the mannose containing bacterial cell wall and the mannose receptor on the macrophage (Mokoena and Gordon, 1985; Ezekowitz and Stahl, 1988). These receptors then allow phagocytosis either by interacting directly with the cytoskeleton or by co-

stimulation of another surface receptor that is able to interact directly with the cytoskeleton.

Receptors for cytokines, lipoproteins, advanced glycosylation endproducts and various glycoproteins all initiate interactions between the macrophage and the receptors ligand to induce internalisation, either to store the particle or to induce a variety of functional responses in the macrophage, including the secretion of cytokines or inhibition factors, and functional changes, such as activation. In this way the macrophage not only aids to "clean up" the extracellular milieu, but also activates and signals bystanding cells. An aspect of these activities is antigen presentation, and it is not surprising that macrophages are excellent antigen-presenting cells, processing various foreign antigens for display by MHC class I and II molecules to appropriate T-cell populations (Adams and Hamilton, 1992; Safley and Ziegler, 1994).

The integrin adhesion receptors are used essentially to aid migration of the cells from the blood stream, through the wall of the blood vessel to the site of inflammation. This will be discussed in more detail in a later chapter. Without these adhesion receptors the cells are powerless to act as defenders at the site of pathology. The various receptors discussed are linked by an intricate network of inter- and intra-cellular communications that are initiated by cells of the immune system, culminating in the activation of the macrophage. Tables 4.1 and 4.2 indicate the complex interactions between the surface receptors, ligands, and the effects of cytokines on macrophage function (Adams and Hamilton, 1992; Crocker and Milon, 1992).

Table 4.1: Macrophage Surface Receptors and their Ligands

(Adapted from Auger and Ross, 1992)

RECEPTORS	LIGANDS
Anticoagulant and coagulant receptors	α1-antithrombin; coagulation factor VII; fibrinogen-fibrin; heparin
Complement receptors	C3; C3b; C3bi; C5a; C1q
Cytokine receptors	MIF; MAF; LIF; CF; MFF; IL-1; IL-2; IL-3; IL-4; IFN-α; IFN-β; IFN-gamma; GM-CSF; M-CSF
Fc receptors	IgG_{2a}; IgG_1; IgG_3; IgA; IgE
Fibronectin receptors	fibronectin
Fucose receptors	fucose
Galactose receptors	galactose
Hormone receptors	angiotensin; insulin; glucocorticosteroids
Lactoferrin receptors	lactoferrin
Laminin receptors	laminin
Lipoprotein receptors	apolipoproteins B and E;
Lipid receptors	PGE_2; LTB_4; LTC_4; PAG
α_2-Macroglobulin-proteinase receptors	macroglobulin complex

Mannose receptors	mannose
Peptide and small molecule receptors	arg-vasopressin; endorphins; H_1; H_2; 5HT; *N*-formylated peptides; substance P; 1,25 Vitamin D_3
Transferrin receptors	transferrin
Scavenger receptor	

ABBREVIATIONS: **C.**, complement; **CSF**, colony-stimulating factor; **GM**, granulocyte macrophage; **H**, histamine; **Ig**, immunoglobulin; **IL**, interleukin; **LIF**, leukocyte migration inhibition factot; **LT**, leucotriene; **MAF**, macrophage activating factor; **MFF**, macrophage fusion factor; **MIF**, macrophage inhibitory factor; **PG**, prostaglandinn; **5HT**, 5-hydroxytryptamine.

Table 4.2: Cytokine Effects on Macrophages

(Adapted from Auger and Ross, 1992)

CYTOKINE	EFFECT ON MACROPHAGE
IL-1	stimulate IL-1; TNF; CSF
IL-3	growth and differentiation of macrophages
IL-4	antigen presentation; fusion; MHC II expression; FcR induction
GM-CSF	activation; growth and differentiation
IFN-α	activation; antiviral activity
IFN-β	activation; antiviral activity
IFN-gamma	MHC II expression; respiratory burst; MFR suppression; antiviral activity
M-CSF	growth and differentiation
TNF-α	IL-1; PAF and PGE_2 release; chemotaxis
TGF-β	deactivation; chemotaxis; growth factor release

ABBREVIATIONS: **GM-CSF**, granulocyte-macrophage colony stimulating factor; **IL**, interleukin; **M-CSF**, macrophage colony stimulating factor; **MFR**, mannose-fucose receptor; **PAF**, platlet activating factor; **TGF**, transforming growth factor; **TNF**, tumor necrosis factor.

4.1.4 Macrophage functions:

The functions of macrophages are varied and related to the role of the organ in which they reside. In most cases, their main function is to act as the first line of defence, phagocytosing and eliminating any foreign invading organisms. Macrophages are attracted to areas of pathology by chemotactic gradients generated by cytokines secreted by damaged and participating cells. These recruited macrophages are activated and have altered cell surface receptor expression, as well as enhanced ability to secrete toxic compounds. Locally activated macrophages will also alter their receptor expression, but tend to be less effective at killing invading pathogens than recruited, monocyte-derived macrophages (Adams and Hamilton, 1992).

Initial interactions are either by direct binding of macrophage surface receptors and pathogen cell envelope adhesins, or following deposition of opsonising agents, such as IgG, C3, or C3bi. The deposition of C3/C3bi on the pathogen surface may be either by the presence of serum, or the active production of complement components by the macrophage. Intracellular pathogens that specifically target macrophages may facilitate opsonisation in order to enhance internalisation. The active induction of complement deposition by pathogens may result from initiation of the classical or alternate complement cascades, depending on the nature of the pathogen surface. Alternatively, some organisms have evolved to express surface receptors that bind directly to macrophage receptors, thereby circumventing the need for prior opsonisation (Mosser, 1994; Speert, 1992). The advantages to pathogens of nonopsonic binding and internalisation are not clearly understood, but speculatively these could include: greater speed of entry; efficient binding even in opsonin-poor

environments; and binding host receptors or receptor epitopes different from those bound by opsonins and which result in a mode of internalisation that favours subsequent survival. These mechanisms of non-opsonic entry are thought to take place mostly in areas with low concentrations of Ig and complement, such as the human bronchoalveolar region, even though alveolar macrophages express FcR and complement receptors. Interestingly, the alveolar macrophage is unique in that it requires specific oxygen tensions to enable effective phagocytosis, which may affect its activity in the granuloma (Reynolds et al., 1973).

4.1.4.1 Genetics of macrophage-dependent host resistance:

The genetic basis of host susceptibility has long been a controversial subject, and studies attempting to distinguish between racial or ethnic factors on the one hand and socio-economic conditions on the other hand failed to establish clearly a pattern of genetic inheritance in humans. Nevertheless, the phagocytic and bactericidal capacity of the human macrophage has been studied in detail, with a view to shedding light on possible genetically determined differences in disease incidence. Animal studies indicate specific relationships between genetic variations and the host immune response when confronted with *M. tb.* In the mouse this is a two phased procedure, with an initial innate immunity that determines the extent to which an infection is established, followed by protective immunity that controls, and may eliminate, the initial infection, and which prevents progression to disease. The innate immunity possessed by some mice to *M bovis* BCG has been traced to a single autosomal dominant gene, the *Bcg* gene, which has been found to be identical to the well characterised *Ity* and *Lsh* genes that provide animals with resistance to *Salmonella typhimurium* and *Leishmania*

donovani. Extensive studies have shown that this gene is expressed in the macrophage and the *Bcg*^{r} (resistant) phenotype is characterised by an enhanced capacity of macrophages to effectively phagocytose and inhibit the intracellular growth of pathogens. A protein known as the natural resistance-associated macrophage protein (NRAMP) is the product of the *Nramp* gene identified as a candidate for the *Bcg* locus (Vidal et al., 1993). It is exclusively expressed in macrophages, and it has been speculated that NRAMP allows nitric oxide to be retained and concentrated in bacilli-containing phagolysosomes of the macrophage, achieving high nitric oxide concentrations that are toxic and lethal to the bacilli (Vidal et al., 1993; Blackwell et al., 1995). Research on human NRAMP is intense, but although a human homologue of the mouse *Nramp* gene has been found, its effect on innate immunity in the human host still has to be proven (Ehlers, 1995; Gros et al., 1981; Buschman et al., 1989; Sadick, 1992; Vidal et al., 1993; Blackwell et al., 1995). With the help of studies such as these the dispute surrounding epidemiological data on populations in old age homes and prisons, which suggest a genetic basis for an increased susceptibility of Black individuals to TB rather than a purely socio-economic basis, may be resolved (Stead et al., 1990; Crowle et al., 1990).

4.1.4.2 Macrophage microbicidal defences:

Once an organism has been internalised, the macrophage has a number of microbicidal mechanisms, both oxidative and non-oxidative, to destroy the particle. The activation of these intracellular defence mechanisms is related, in part, to the pathway of entry. If bacteria enter the macrophage via the Fc receptor, they initiate the release of large amounts of oxygen radicals and arachidonic acids, commonly referred to as the

phagocytic respiratory burst. This enables the macrophage to attack the phagosomal contents and thereby destroy the organism. On the other hand, organisms that enter via the C3-complement receptor interaction are able to evade the respiratory burst and may manipulate the intracellular environment for their own benefit (Speert, 1992). Some organisms may disarm the macrophage's second line of defence, by inhibiting, inter alia, lysosome-phagosome fusion, thereby avoiding contact with lysosomal contents, which consists of a number of microbicidal and fungicidal hydrolases and peroxidases. Other pathogens do not attempt to halt phagosome-lysosome fusion, preferring instead to survive in this compartment, and may even utilise the low pH to promote growth and differentiation. The non-oxidative, lysosomal mechanism of intracellular bactericidal activity is constitutively present in the macrophage and does not require activation, but not all resident macrophages contain the full complement of enzymes required for effective bactericidal activity, as, for instance, the alveolar macrophage (Stokes et al., 1993; Sadick, 1992).

4.1.4.3 Macrophage-derived cytokines and inflammation:

Macrophages, in order to react effectively to noxious stimuli and invasive pathogens, must be able to respond rapidly to chemotactic signals, such as tuberculin or pertussis toxin. In addition to migration, quiescent macrophages must be activated to enhance their microbicidal and antigen-presenting capacities. Activation is achieved by various cytokines, of which interferon-gamma (IFN-gamma) is the most important. The cytokines initiate a cascade of intracellular events that allow the macrophage to migrate and interact with the organism. This interplay between signals and responses is carefully regulated so as to control the effects and terminate the responses. The

most carefully studied area of cellular regulation is the protein kinase C pathway, which regulates both intra- and inter-cellular responses. Macrophages not only receive and respond to cellular signals, but also directly interact with and stimulate other cells, by secreting a panel of macrophage-derived cytokines (often referred to as inflammatory cytokines), including IL-1, IL-6 and TNF-α. Table 4.3 describes a number of macrophage secreted components and their functions (Celada and Nathan, 1994; Reiner, 1994).

Similarly, the ability of a lymphocyte to respond to a chemotactic signal will often depend upon the presentation of a foreign antigen on the macrophage cell surface. Antigen processing and presentation is a major function of the macrophage. This may either take the form of Class I-restricted antigen presentation, where the antigen is derived from the cell cytoplasm, transported into the endoplasmic reticulum (ER), and then transferred to the surface, or as Class II-restricted antigen presentation, in which exogenous antigens are presented after endocytosis and/or phagocytosis (Safley and Ziegler, 1994).

Apart from the protective role that macrophages play in the body, they are intimately involved in the acute phase response to disturbances in the homeostasis of the body. Hepatic monocyte-macrophage products are central to the effective interaction between cells during the acute phase response and lead to the common symptoms associated with acute and chronic disease, such as fever and enhanced protein catabolism, often resulting in cachexia. Macrophages also aid in tissue damage repair and the disposal of degraded material, both self and non-self, by recognising and

Table 4.3: Products Secreted by Macrophages

(Adapted from Auger and Ross, 1992)

PRODUCT	EXAMPLES
Enzymes	
lysozyme	
lysosomal hydrolases	proteases,lipases, phosphatases, sulphatases
neutral proteases	collagenases, elastases, angiotensin convertases, cytolytic proteinases
lipases	lipoprotein lipase, phospholipase A2
Enzymes & Cytokine Inhibitors	
protease inhibitors	α_2-macroglobulin, plasminogen activator inhibitor, collagenase inhibitor
phospholipase inhibitor	
IL-1 inhibitors	
Complement Components	
classical pathway	C1,C4,C2,C3,C5
alternative pathway	factors B and D
active fragments	C3a,C3b,C5a,Bb
inhibitors	C3b inactivator
Reactive Oxygen Intermediates	superoxides, hydrogen peroxide, hydroxyl radical
Coagulation Factors	
prothrombin activator	
factors	II, VII, IX, X, XIII
plasminogen activators	
plasminogen inhibitors	
Cytokines	IL-1, IL-6, IL-8, TNF-α, IFN-α, IFN-β, fibroblast and platelet-derived growth factors, GM-CSF, M-CSF, erythropioetin, angiogenesis factor
Others	thrombospondin, fibronectin, liprofectin, transferrin, ferritin, haptoglobin, uric acid, apolipoprotein E, neopterin

phagocytosing old or damaged cells (Pollock and Etzioni, 1992).

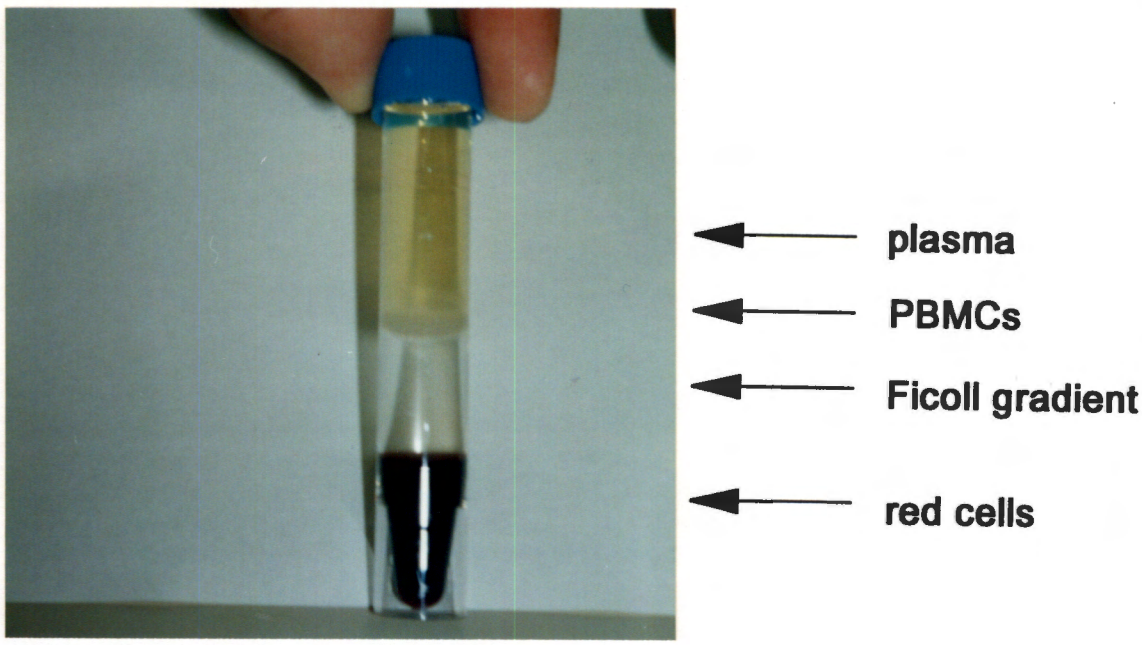
Despite their dominant and vital protective role in the body, macrophages may also become defective and thereby play an integral part in a number of autoimmune diseases, an example of which is sarcoidosis, an inflammatory disease of the lungs of unknown aetiology, which results in the formation of granulomas similar to tuberculous granulomas. It is thought that this irregular response is due to the inappropriate activation of the immune system by macrophage-secreted cytokines, resulting in the formation of "protective" granulomas. These irregularities are few in comparison to the effective "housekeeping" functions that the macrophages perform and their crucial role in first-line host defence, without which the body certainly would not function effectively and coordinatedly in the maintenance of homeostasis and in protection against pathogen invasion (Pollock and Etzioni, 1992).

4.2 MATERIALS AND METHODS:

4.2.1 Isolation and Culture of Monocyte-derived Macrophages (MDMs):

Forty millilitres of peripheral blood were drawn directly into 4 heparinised 10ml glass tubes, from a Mantoux negative individual. An additional 20 ml of blood was drawn, in a glass clotting tube, to provide autologus serum. Blood was processed within 2 hours. The heparinised blood was diluted 1:2 with room temperature PBS, in 12ml

Figure 4.3: Ficoll sedimentation gradient of isolated peripheral blood mononuclear cells



Fresh heparinised blood, diluted with PBS and layered onto a Ficoll density gradient results in the separation of the blood cells, as indicated.

plastic tubes. Seven millilitres of the diluted blood were then carefully layered onto 3 ml of Ficoll Histopaque (Sigma) in 10ml plastic tubes. The tubes were centrifuged at 1800 rpm for 20 minutes to generate a sedimentation gradient, as indicated in Figure 4.3. The peripheral blood mononuclear cells (PBMCs) are at the interface, just above the Ficoll layer, and were aspirated with a sterile Pasteur pipette and transferred into a clean 10ml tube. The PBMCs were washed three times in PBS, with pelleting of the cells at 1600 rpm for 10 minutes between washes. The PBMCs were counted on a Haemocytometer and resuspended in 20% autologus fresh serum and RPMI-1640 to obtain approximately 5 x 10^6 cells/ml. Two millilitres of the suspension were seeded per well in a 6-well plate and incubated at 37°C with 5% CO_2 for 1½ hours, to allow adherence of the monocytes to the plastic. The non-adherent PBMCs were washed off with RPMI-1640 and the cells were reincubated as before for a further 3 days. On day 3 the culture supernatant was aspirated and saved, the cells washed once with RPMI-1640 and refed with 10% autologus serum in RPMI-1640, supplemented with 25% culture supernatant. This culture supernatant was, centrifuged to remove all the non-adherent cells, and referred to as conditioned medium as it contains cytokines and components secreted by the PBMCs during maturation that aid the monocytes in maturing further. Only after day 6 were the monocytes classified as MDMs and used in experiments as macrophages, for reasons described in Figures 4.1 and 4.2.

4.2.2 Seeding of MDMs:

MDMs were lifted from the plastic tissue culture dish by adding 1 ml of cold 5mM EDTA/HBSS solution per well and incubating the cells at 37°C with 5% CO_2 for 5 minutes. The cells were then washed off the plastic with a Gilson P1000 pipette and resuspended in 10 ml of PBS. The cells were centrifuged at 1600 rpm for 10 minutes to remove the EDTA and the pellet resuspended in 1 ml of medium. The cell suspension was quantitated by diluting the cells in Trypan blue and counting on a Haemocytometer, after which they were seeded onto 12mm glass coverslips at a ratio of 1 x 10^5 cells per coverslip in a 24-well tissue culture plate. The cells were incubated in 10% autologus, fresh serum in RPMI-1640, at 37°C with 5% CO_2 for 4 hours, before being used in an assay. Cells were seeded in duplicate or triplicate per experiment.

The plated cells were then washed and incubated in the desired medium appropriate for the assay conditions. These were either with or without 10% fresh, autologus serum in RPMI-1640. No antibiotics were present in these media.

4.2.3 Infection Ratios of MDMs:

Mycobacterium tuberculosis (H37Rv), cultured as described in Chapter 3, and diluted to McFarland's standard 0.5 (1.5 x 10^8 organisms/ml), was added in 50µl aliquots to the various wells, to obtain an infection ratio of approximately 50:1 bacteria to cell. The tissue culture plates were swirled gently to distribute the bacteria, and incubated at 37°C, 5% CO_2 for 20 minutes to 2 hours, after which the non-adherent bacteria were

removed by gentle washing with PBS, and the cells reincubated in the appropriate media for up to 24 hours.

4.2.4 Fixing and Staining of Infected Cells:

After the required incubation period the cells were washed at least three times with PBS to remove any non-adherent bacteria, and then either fixed in 2.5% glutaraldehyde (GA) and kept at 4°C, or air dried and heat fixed. The GA-fixed coverslips were washed again with PBS to remove any residual GA and flooded with a 1.4 mg/10 ml solution of acridine orange for 1 minute at room temperature, before being washed with PBS. The extracellular fluorescence was then quenched with a 5 mg/10 ml crystal violet solution for 30 seconds at room temperature. Cells were washed again and fixed onto a glass microscope slide for examination by fluorescence microscopy at an excitation wavelength of 350 nm, as indicated in Figure 4.4.

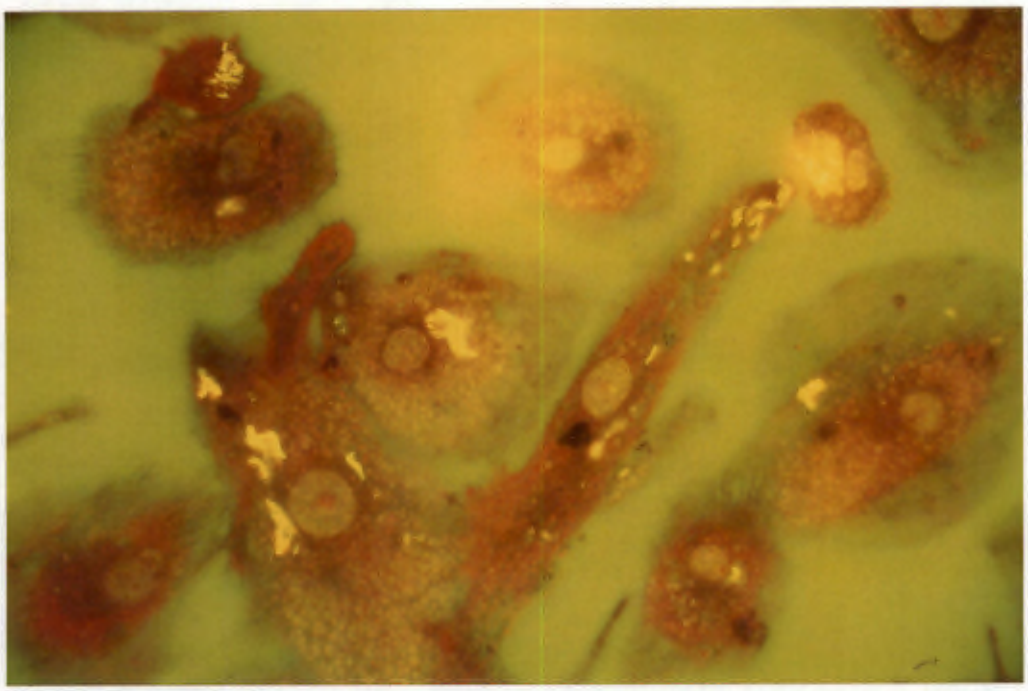
The air-dried/heat-fixed slides were stained with Ziehl Neelsen stain as described in Addendum A.1.15., and viewed with light microscopy as indicated in Figure 4.5.

4.3 RESULTS:

4.3.1 Growth and Recovery Rates of MDM:

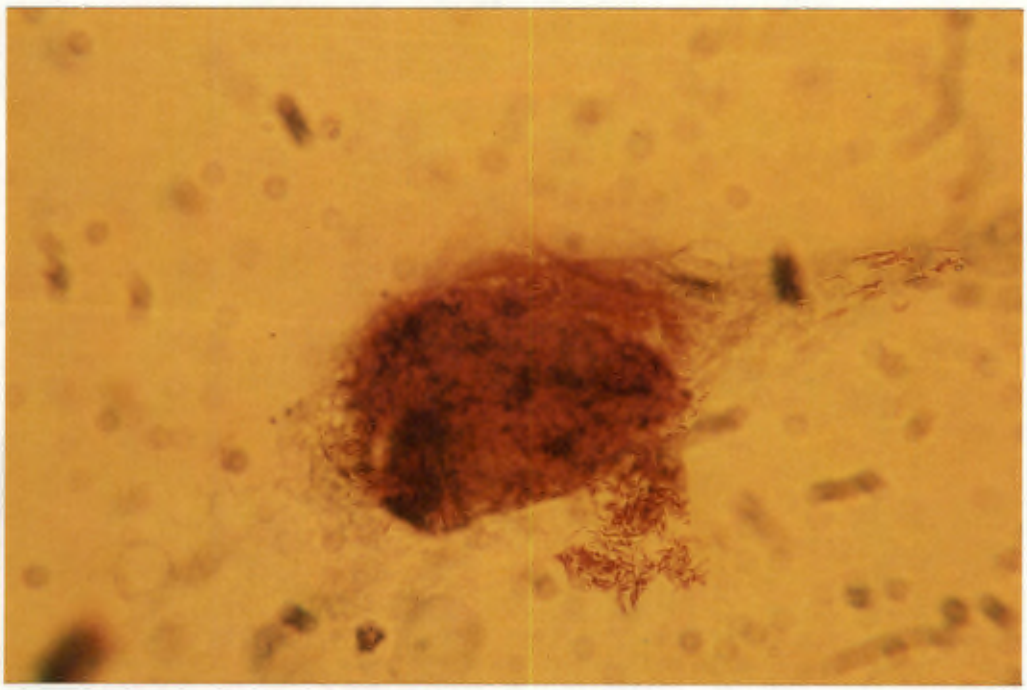
All MDMs were culturable for a period of 2-3 weeks, before they were no longer able to phagocytose and adhere. Most cells were used in assays before 2 weeks, during which time the media were changed every three days.

Figure 4.4: Acridine orange staining of *M. tb.* associated with MDMs

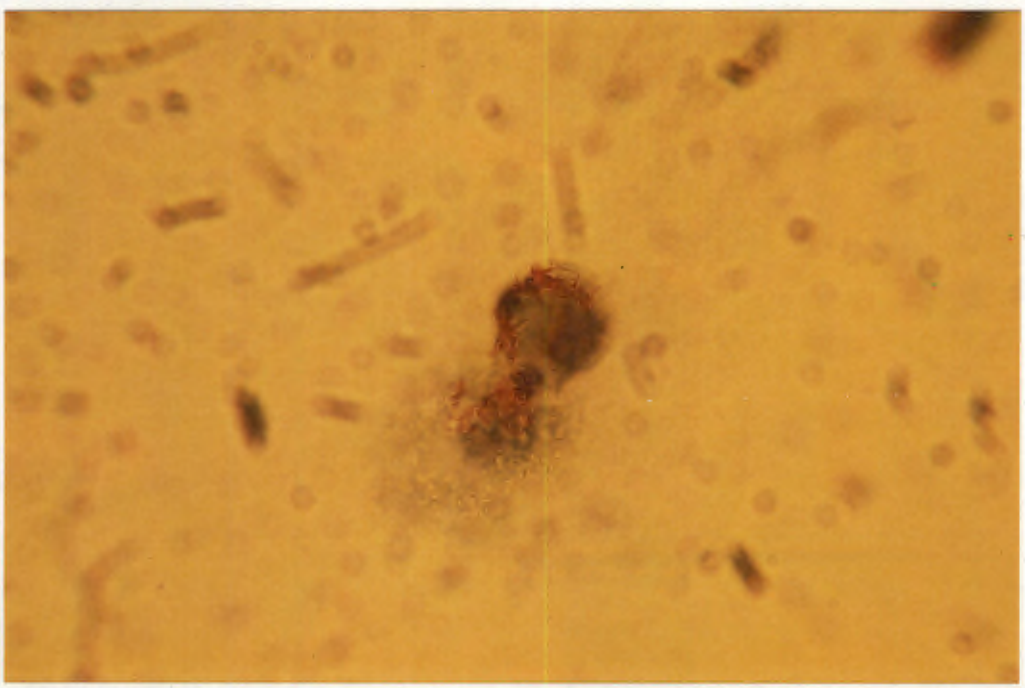


M.tb infected MDMs were fixed with glutaraldehyde, stained with acridine orange and quenched with crystal violet, to allow easy enumeration of infected cells with fluorescent microscopy.

Figure 4.5: Ziehl-Neelsen staining of phagocytosed *M. tb.*



A different method of staining *M. tb.* infected MDMs, that aid identification of any co-contaminating pathogens.



The number of cells recovered from each batch of fresh blood varied from batch to batch, but on average 5% of the total white blood cell (WBC) count represented culturable monocytes.

4.3.2 Association of *M.tb* with MDMs:

The MDMs were infected with H37Rv as described above, and the number of bacteria associated with the cells enumerated by both ZN staining and fluorescent microscopy after acridine orange staining. The number of bacteria associated with each cell was expressed as a percentage of the total number of cells counted per coverslip, with no attempt being made to distinguish between extracellular and intracellular bacteria. The infections were performed in the presence and absence of fresh serum, for 3 and 24 hours.

The cells were infected at a ratio of 500:1 and at 50:1, bacteria per cell. The cells were incubated in appropriate media for the experiment. Cells infected at greater than 100:1 bacteria to cell ratios, rapidly phagocytosed the bacilli, with resultant lysis of the overfilled macrophages. These experiments revealed only cellular debris and numerous extracellular bacteria.

Macrophages infected at a 50:1 ratio showed good phagocytic ability, with on an average 55 ± 8% (4.5 bacteria / cell) of the cells associated with bacteria after 3 hours and 80 ± 3% (6.2 bacteria / cell) after 24 hours incubation in medium containing 10% fresh autologus serum. In contrast, MDMs infected at a 50:1 ratio in the absence of serum indicated an association of 23 ± 9% (3.9 bacteria / cell) after 3 hours and 57 ±

7% (5.7 bacteria / cell) after 24 hours. Results are from counting of 100 MDMs on between 2 and 5 separate coverslips. The addition of 10% fresh FCS in the assays gave results similar to those with autologus serum.

Heat inactivation of the autologus serum, at 56°C for 30 minutes, resulted in a modest reduction in bacterial association, when compared to the results obtained with fresh serum. Thus, of MDMs infected at a 50:1 ratio, 37 ± 4% (3.6 bacteria / cell) were associated with bacteria after 3 hours (33% reduction), and 64 ± 6% (5.2 bacteria / cell) cells were associated with bacteria after 24 hours (20% reduction).

4.4 DISCUSSION:

The functions of the macrophage CR3, together with other surface receptors, as an integral component of macrophage defence mechanisms are well established. However, exactly how CR3 interacts with pathogens, notably *M. tb.*, in a complement-deficient environment has still to be elucidated. It has been suggested that bacteria that enter via this pathway are able to avoid the respiratory burst and this constitutes a specific survival strategy.

To investigate the role of serum opsonins in *M. tb.* infections, MDMs were infected at various bacterial to cell ratios and serum conditions. The results we have obtained are comparable with those reported in the literature (Stokes et al., 1993; Hirsch et al., 1994; Simms et al., 1989) and provide us with a reproducible, standard infection assay. MDMs infected at ratios greater than 100:1 bacteria to cell do not provide useful data, as the vast majority of the cells loaded with bacteria lyse within 24 hours. We found that the association of MDMs with bacilli was similar in the presence of media

supplemented with fresh, autologous serum or media supplemented with fresh FCS, suggesting that fresh FCS constitutes a source of opsonins that is adequate for interaction with human MDM receptors, including CR3.

Based on these results, we have relied mainly on fresh FCS in later assays using transfected CHO cells (Chapter 6). The results obtained in the absence of serum similarly agree with published reports and provide *in vitro* evidence for the phagocytic ability of macrophages in complement-deficient environments (Stokes et al., 1993). Although only 23% of the cells were associated with bacteria after a 3-hour incubation, most of these cells bound large numbers of bacteria, which was enhanced further after incubation for 24 hours. The number of bacteria per cell in assays devoid of serum were less than in the presence of serum, whereas assays performed with heat-inactivated serum gave intermediate values. Most MDMs had an average of 3-10 bacteria per cell in the presence of serum, with similar results of 1-10 bacteria per cell obtained with heat-inactivated serum, as compared to 1-5 bacteria per cell in the absence of serum. All assays had enhanced association after 24 hours.

Interestingly differences arose from the mode of preparation of MDMs for the infection assays. When MDMs were seeded, cultured and infected on the same glass coverslip, they showed a lower association with bacteria (36 ± 4% in the presence of serum and 15 ± 2% in the absence of serum, after a 3-hour incubation) than when they were seeded and cultured in plastic culture dishes first, before detachment and seeding onto glass coverslips prior to infection (55 ± 8% in the presence of serum and 23 ± 9% in the absence of serum, after a 3-hour incubation); these differences were noted both in the presence and absence of serum. These data suggest that detachment and reattachment may in some way activate MDM receptors, thereby enhancing the

interaction with the *M. tb.* cell wall, including in the absence of complement. These results corroborate recent data by Stokes et al., 1993, which document the importance of the phenotype and activation state of murine macrophages with regard to complement-independent phagocytic ability.

There are a number of factors that influence the number of cells isolated, the adherence capacity, and the viability of the cells, many of which are difficult to control. These include the immune status of the individual at the time of venesection as well as the lipaemic state of the serum. Care must be taken to obtain age- and sex-matched donors for this purpose.

The use of MDMs *in vitro* as representative of *in vivo* resident macrophages has advantages and disadvantages, such as the procurement of macrophages representative of the *in vivo* conditions, for example alveolar macrophages, the culture conditions required for these cells *in vitro* and the lifespan of the harvested cells in culture. Whatever their final merits, they do provide a useful reference or baseline for *M. tb.*-host cell interactions. Our main aim in using the macrophage system was to set up a reproducible, standard infection assay as a reference to evaluate subsequent studies involving the infection of CR3-expressing CHO cells with *M. tb.*

There are many unanswered questions regarding the role of CR3 in binding *M. tb.* and its dependence on complement, as well as the ability of the human macrophage to kill *M. tb.* Some of these questions are difficult to answer with studies based on *in vitro* infections of macrophages, including MDMs, because of complications arising from the large number of cell surface receptors and functions of the macrophage. It was for this reason that we decided to construct a non-phagocytic cell line that expressed the

receptor of interest, CR3. In this way we are able to study this receptor, in isolation from the other macrophage receptors and functions.

CHAPTER FIVE

CONSTRUCTION AND FUNCTION OF CR3-EXPRESSING CELLS

As explained in detail in Chapter 4 , *M.tb*. binds to a number of different cell-surface receptors on mononuclear phagocytes. We have hypothesised that the binding to CR3, in particular, confers specific advantages and facilitates the survival and growth of *M.tb* after invasion of mononuclear phagocytes. To enhance our understanding of the interaction between *M. tb.* and CR3, we decided to clone the receptor into a non-phagocytic cell, thereby avoiding, inter alia, the possible interference of other macrophage receptors and the endogenous synthesis of complement components. The interaction with and manipulation of CR3 by pathogens, to exploit host cells, is not novel, suggesting that *M. tb.* may make use of a similar mode of entry. The following chapter provides a broad overview of the functions and structure of integrins and describes the subcloning and transfection procedure for human CR3.

5.1 INTEGRINS: STRUCTURE AND FUNCTIONS

5.1.1 Introduction:

During invasion by pathogens, professional phagocytes, including macrophages and neutrophils, are activated to defend the host against invasion, progression of an

infection to disease, and dissemination. For this reason macrophages, the first line of defence in tissues, have evolved to display a number of cell surface receptors that will aid them to recognise and react to foreign bodies, usually leading to the engulfment and destruction of the pathogen in the immuno-competent host. But it is exactly these cell surface receptors that the facultative, intracellular pathogens utilise to manipulate the host cells to benefit their own ends. The most commonly manipulated cell surface receptors are the complement receptors CR1, CR3, and CR4, which interact with opsonised particles. Of these, the CR3 and CR4 belong to the highly conserved family of heterodimeric, glycoprotein cell adhesion molecules, otherwise known as Integrins, which are responsible for a number of cell-cell and cell-matrix interactions that determine the fate of most cells. The receptors were named integrins to describe their function of integrating the intracellular cytoskeleton with the extracellular matrix (Haas et al., 1994). CR1, on the other hand, is also closely involved with opsonised phagocytosis, but is characteristically a single subunit glycoprotein with four allotypes that are known to interact mainly with complement component C3b, but also with C4b and C3bi, and induces phagocytosis in this manner in activated macrophages (Ross and Medof, 1985).

5.1.2 Cellular Distribution of Integrins:

Integrins are universally distributed throughout host tissues, with individual cells expressing a range of integrins in varying numbers and proportions. Most tissues express the ß1 family of collagen adhesion receptor, namely α1ß1, α3ß1 and α6ß1, as well as the fibronection binding receptor, α5ß1, which is less well expressed.

Leukocytes of the immune system are, so far, the sole expressors of the ß2 integrin family, αLß2, αMß2 and αXß2. These cells also express collagen receptor α1ß1 and α2ß1, and the fibronectin receptor α5ß1, to aid in cell-matrix interactions. Most of the integrins do not function independently, but rather synergistically with other adhesion molecules, to achieve a complex integration of adhesion events (Albelda et al., 1992; Arnaout, 1990) (Table 5.1).

5.1.3 Biosynthesis and Structure of Integrins:

The α and ß integrin subunits have separate precursors, which are independently synthesised and released from the Golgi, after which they associate in a non-covalent manner before reaching the cell surface; stable expression of each subunit depends on assembly into a heterodimer. The associated heterodimer now has an increased molecular mass and takes about 50-80 minutes to reach the cell surface. The α subunit usually dictates the glycosylation pattern of the ß subunit, but this may be altered according to the cell type or ligand specificity required, thereby making integrins adaptable for a variety of adhesive interactions (Arnaout, 1990).

Complete heterodimers are stored in their inactive forms in secondary and tertiary granules in granulocytes, and in intracellular vesicles and peroxidase-negative granules in monocytes. Once the cells are activated in response to a stimulus, defined intracellular events take place and integrin surface expression is upregulated within minutes by means of translocation of the intracellular granules and resultant fusion with the plasma membrane. This occurs in most cells except resident tissue macrophages, which contain no intracellular pools (Arnaout, 1990; Hynes, 1987; Hynes, 1992).

Table 5.1: The Integrin Receptor Family

SUBUNITS	ALTERNATE NAMES	CELLULAR DISTRIBUTION	I DOMAIN
α1β1	CD-/CD29; VLA-1	Fibroblasts; connective tissue; basement membrane associated; activated T & B cells	Yes
α2β1	CD49b/CD29; VLA-2; gpIaIIA; ECMRII	Platelets; fibroblasts; connective tissue; endothelial, epithelial, LAK and activated T cells	Yes
α3β1	CD-/CD29; VLA-3; ECMRI	Epithelial and LAK cells; fibroblasts; connective tissue; monocytes	No
α4β1	CD49d/CD29; VLA-4; LPAM-1	Neural crest, B & T cells; large granular lymphocytes; fibroblasts; connective tissue	No
α5β1	CD-/CD29; VLA-5; FNR; gpIc/iiA; ECMRV	Fibroblasts; connective tissue; platelets; monocytes; platelets; thymocytes; epithelial, endothelial, LAK and T cells	No
α6β1	CD49f/CD29; VLA-6; gpIc/IIa	Platelets; T cells	No
α7β1			
α8β1			

αVβ1	CD51/CD29	Neural crest; fibroblasts; connective tissue	No
αLβ2	CD11a/CD18; LFA-1	B & T cells; monocytes; granulocytes	Yes
αMβ2	CD11b/CD18; MAC-1; CR3	Monocytes; granulocytes	Yes
αXβ2	CD11c/CD18; p150,95	Monocytes; granulocytes	Yes
αIIbβ3	CD41/CD61; gpIIb/IIa	Platelets; endothelium	No
αVβ3	CD51/CD61; VNR	Endothelium; monocytes; activated B cells	No
αRβ3		PMN; monocytes	No
α6β4	CD49f/CD-; αEβ4	Epithelial cells	No
αVβ5	CD51/CD-	Carcinoma & epithelial cells; monocytes; connective tissue; fibroblasts	No

ABBREVIATIONS: CD, cluster denomination; **VLA,** very late antigen; **ECM,** extracellular matrix; **LAK,** lymphokine-activated killer; **FNR,** fibronectin receptor; **LFA,** leukocyte functional antigen; **VNR,** vitronectin receptor; **PMN,** polymorphonuclear leukocyte.

Integrin αß heterodimers consist of large globular N-terminal extracellular domains, which function as the ligand-binding domain; a short highly conserved transmembrane region; and a short cytoplasmic domain, which interacts with the cytoskeleton and intracellular signalling pathways (Corbi et al., 1988) (Figure 5.1a).

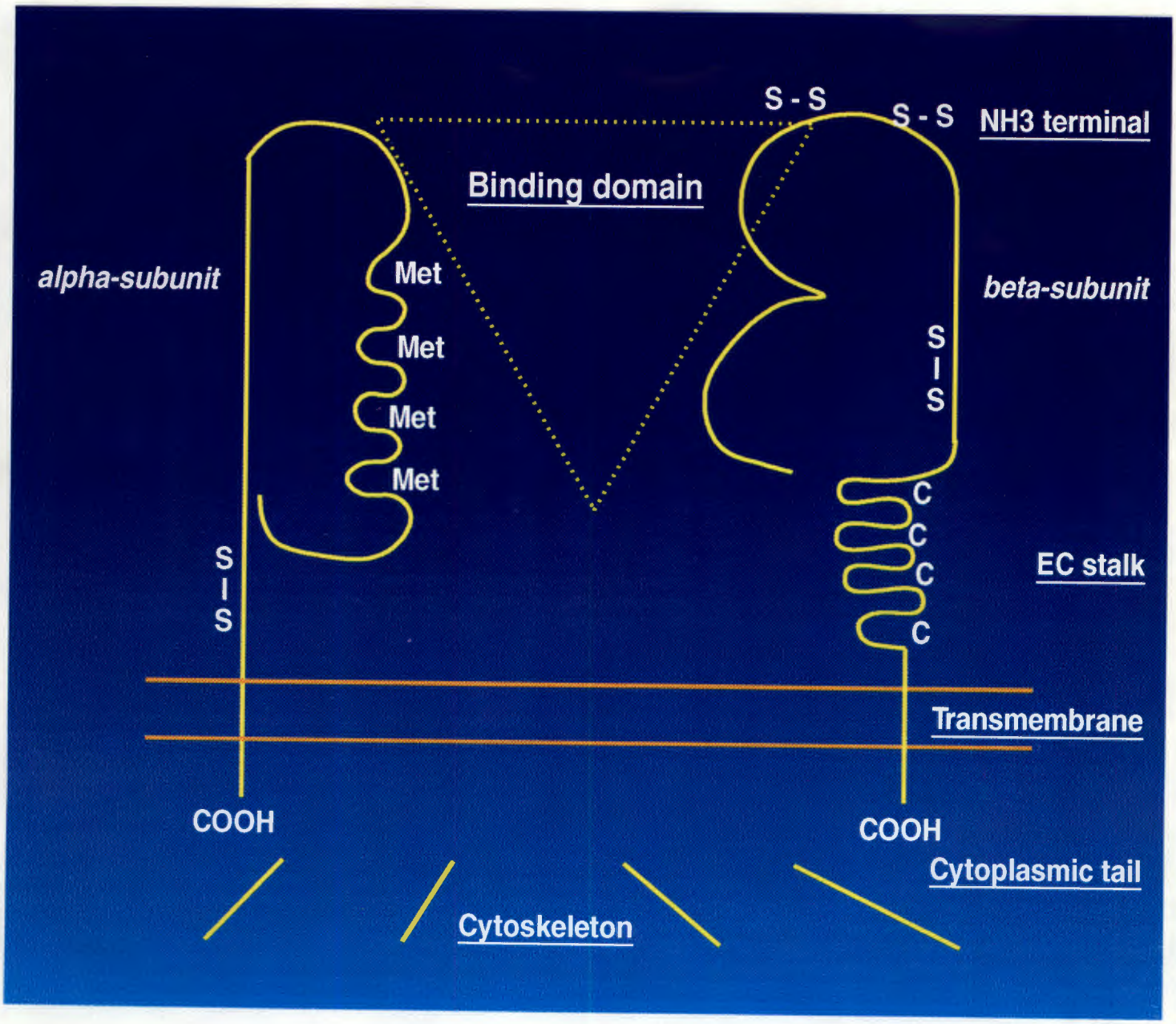
Each ß subunit contains 56 cysteine residues distributed between 4 cysteine repeats in the extracellular stalk domain, with an internal disulphide bond that serves to efficiently expose the ligand-binding site contained in the 40-50 kDa N-terminus (Figure 5.1a).

The α subunit in turn is made up of 7 homologous tandem repeats of ±60 residues, with repeats 5 to 7 each containing the nanopeptide providing the metal-binding region of the extracellular domain (Arnaout, 1990). The disulphide bond in the stalk region of the extracellular domain provides stability and resistance to proteolytic cleavage, by introducing folding of the subunits. The cytoplasmic region is short and utilised for phosphorylation events, which link extracellular with intracellular events and vice versa, thereby establishing a communication system between the inside and outside of the cell (Figure 5.1a).

Recent studies have indicated that the ß2 subunit colocalises with talin in the cytoplasmic domain, suggesting either an indirect interaction, via the α -subunit and then the ß-subunit, or direct interaction, via the ß-subunit, with the cytoskeleton, enabling successful participation in phagocytosis (Arnaout, 1990).

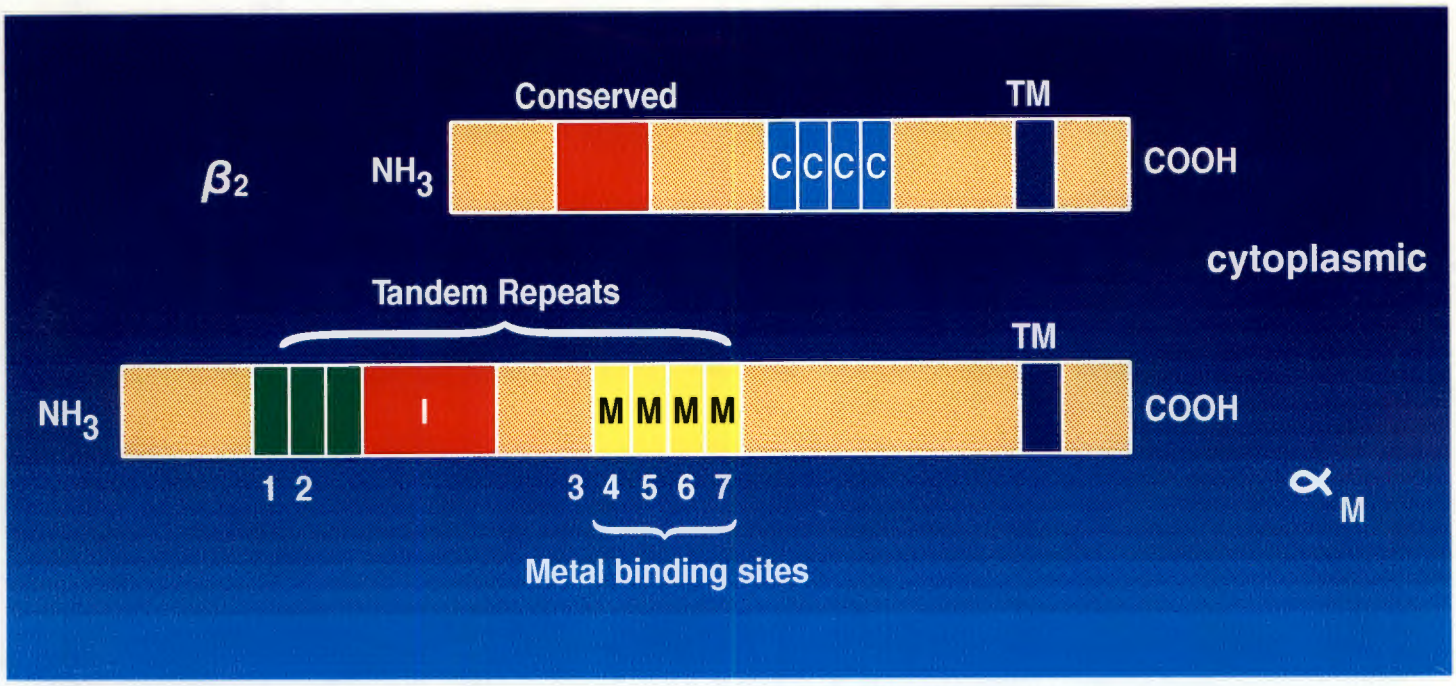
In the ß1 and ß2 families of integrins an additional 200-amino acid fragment has been inserted between the last metal-binding region and the N-terminus of the α subunits. This domain is commonly known as the I or A domain, and has direct effects on the specificity and affinity of ligand-binding functions (Figure 5.1b). Point mutations in this region result in an inability of the cells to adhere, diapedese and migrate in

Figure 5.1a: Schematic structural features of integrins



Abbreviations: **S-S**, disulphide bonds; **Met**, metal-binding region

Figure 5.1b: Novel and conserved domains of the β2-integrin family



Abbreviations: **TM**, transmembrane domain; **I**, I or A domain

response to chemotactic factors released by pathogen-invaded cells, as demonstrated in the life-threatening Leukocyte Adhesion Deficiency (LAD) disease (Arnaout et al., 1991).

Truncated subunits have been shown to be conformationally active in epitope-specific monoclonal antibody (MAb) binding studies, even without cytoplasmic and transmembrane regions, implying that these regions are not required for constitutive activation. This may be true for certain epitopes, but does not reflect the entire spectrum of epitopes that could be involved in cell-ligand interactions. This would depend upon cell signalling events mediated via the cytoplasmic tail, to enable conformational changes and expose the required epitopes, as demonstrated by the ß1 and ß3 subunits in association with their ligands (Hynes, 1992; Haas et al., 1994).

5.1.4 Functions of Integrins:

To enable cells of various tissues to function effectively in response to stimuli, they need to be in constant contact with the extracellular matrix (ECM) and other cells. This in turn leads to activation of the cell and the upregulation of integrins to enable intercellular communication and ligand interactions. This function is not only important during embryogenesis, cellular growth and differentiation, malignant transformation, and hemostasis, but is also centrally involved in the host defence against pathogen infections and tissue destruction. During an immune response, integrins help by mediating chemotaxis, phagocytosis, and degranulation; the latter is accompanied by the release of proteolytic enzymes, superoxides and oxygen radicals, which disable the invading pathogen but also cause extensive tissue destruction. The

tissue destruction is limited to the area of inflammation by the immune cells adhering to the ECM via their integrins, thereby containing the area of destruction (O'Toole et al., 1994).

Recent studies using various chimeras of integrins have indicated that the cell type in which a receptor is expressed has a distinct effect on the function and activation of the receptor, as does the cytoplasmic tail with its conserved tyrosine, threonine and serine residues that are phosphorylation dependent (Williams et al., 1994; Pacifici et al., 1994), even though this region is 180 Ä away from the ligand binding site (O'Toole et al., 1994).

5.1.5 Ligands of Integrins:

Integrin ligands are protein, carbohydrate or lipid molecules that interact with defined epitopes on the integrin to evoke a specific response (Hynes, 1992). Individual integrins are capable of interacting with more than one type of ligand, but different integrins also interact with the same ligand by recognising separate motifs specific for various receptors. For example, fibronectin can be recognised by α 5ß1 and α 4ß1, but they interact with the RGD and ELDV motifs, respectively. The activation state and the type of receptor expressed, as well as the cell type in which it is expressed, will dictate the epitopes exposed during various forms of stimulation. In this way the cell can regulate the interaction between itself (via the integrin) and the ligand and alter the adhesive properties by selective expression. Similarly, the ligand must be in the correct conformation to optimise presentation of the recognition sequence (Haas et al., 1994).

A number of binding sites have already been identified and are steadily increasing, as more molecules are being explored. Known amino acid motifs of various ligands are

Table 5.2: Ligands of the Integrin Receptor Family

INTEGRINS	LIGANDS	BINDING SITE
α1β1	Laminin; collagen	-
α2β1	Laminin; collagen; Fn; echovirus I	DGEA (collagen type I)
α3β1	Laminin; collagen; epilgrin; Fn	RGD?
α4β1	Invasin; VCAM-I; Fn	EILDV
α5β1	Denatured collagen; Fn	RGD
α6β1	Laminin	
α7β1	Laminin	
α8β1	?	
αVβ1	Vitronectin; Fn?	RGD
αLβ2	ICAM-1; ICAM-2	
αMβ2	C3bi; Fx; fibrinogen; ICAM-1; LPS; gp63; FHA; β-glucan	RGD?
αXβ2	Fibrinogen; C3bi	RGD?; GPRP
αIIbβ3	Fibrinogen; Fn; von Willebrand factor; collagen; vitronectin;*Borrelia burgdorferi*	RGD; KQAGDV
αVβ3	Vitronectin; fibrinogen; von Willebrand factor; thrombospondin; Fn; osteopontin; collagen; denatured collagen; HIV *tat* protein	RGD
αRβ3		

α6β4	Laminin?	
αVβ5	Vitronectin; Fn; HIV *tat* protein	RGD
αVβ6	Fn; cytoactin	RGD
α4β7	Fn (V25); VCAM-1; mucosal ACAM-1	EILDV

ABBREVIATIONS: Fn, fibronectin; **VCAM,** vascular cell adhesion molecule; **ICAM,** intercellular adhesion molecule; **C3bi,** preoteolytic fragment of complement component C3; **Fx,** factor X; **LPS,** lipopolysaccharide; ***FHA,*** filamentous haemagglutinin; **ACAM,** addressin cell adhesion molecule; **RGD,** Arg-Gly-Asp; **KQAGDV,** Lys-Gln-Ala-Gly-Asp-Val; **DGEA,** Asp-Gly-Glu-Ala; **EILDV,** Glu-Ile-Leu-Asp-Val; **GPRP,** Gly-Pro-Arg-Pro.

well recognised and studied, as indicated in Table 5.2. These ligands tend to interact with related integrins, as demonstrated by the RGD-binding integrins, which are closely related and contain cleaved α subunits and no I domain (Hynes, 1992).

On further exploration of the I domain, it was noted that the ligands C3bi, fibronectin, ICAM-1 and factor X all bind to different epitopes of the I domain, but require the N-terminal and the metal binding site to be in the correct conformation to bind efficiently (Diamond et al., 1990). Any small deletions or substitutions in this highly conserved I-domain region lead to the abolition of C3bi binding, making it essential for ligand binding. This is further complicated by the requirement for physiological levels of the divalent cations calcium, magnesium, and manganese, which suggests that activation of the epitopes is regulated by inside-out signalling from the cell, as this process is dependent on the influx of calcium and other divalent cations into the cell, to initiate intracellular signalling pathways for the stabilisation of the integrin conformation (Michishita et al., 1993; Graham and Brown, 1991).

Integrins part take in a number of other cellular functions, ranging from acting as co-stimulatory receptors - which will activate cells to produce cytokines or initiate a respiratory burst, as demonstrated by neutrophils upon engagement of the ß2 integrin (Ross et al., 1985) - to contributing to abnormal cell differentiation and gene expression displayed in malignancies. The response of integrins to extracellular stimuli culminates in a cascade of intra- and inter-cellular communication events, mediated by changes in the intracellular milieu, in turn caused by the secretion of phospholipases, increases in intracellular calcium, and activation of protein kinase C (PKC). The extent to which these events affect the cellular behaviour will depend

upon the avidity of the initial ligand-integrin interaction and the ability of the cell to respond to the subsequent signals.

The field of intra- and inter-cellular signalling via integrins is in its infancy, but already great headway has been made in this area. Crosslinking of integrins by ligands or MAbs activates protein tyrosine kinases which in turn allows clustering of the protein kinases in submembranous patches where they are available for activation or reaction with substrates. Such intracellular events are initiated, for example, by the extracellular binding of fibronectin to the fibronectin receptor, which initiates the signalling cascade and activation of the cytoplasmic tail, which in turn activates the receptor to enable various functions, such as the internalisation of bound particles by manipulation of the cytoskeleton. The reverse may also occur, where intracellular signalling activates the cytoplasmic tail in turn to activate the integrin, as seen with PMA stimulation. These two types of signalling are known as outside-in and inside-out signalling and keep the cell in constant communication with the extracellular environment.

This implies that a complex network of information is mediating through the cell at points of adhesion, and that the cell is constantly "aware" of extracellular events (Hynes, 1992).

5.1.6 Leukocyte Adhesion Molecules - CD11/CD18 Family:

Among the leukocyte adhesion molecules (Leu-CAMs; CD11/CD18 complex) the CD11b and CD11c subunits are 63% identical, which is considerably higher than the homology between α subunits in general (23%); this similarity between CD11b and

CD11c extends to their functional homology as well. Genetically, the subunits are found on chromosomes 16p11-p11.2 and 21.q22 for the α and ß subunits, respectively. This colocalisation of the α subunit with CD43 and protein kinase C on chromosome 16, and the ß subunit with Down's Syndrome on chromosome 21, may explain the why there is an over-expression of the ß subunit in Down's Syndrome patients and why interactions between the α subunit and PKC or CD43 are so common (Arnaout, 1990).

As with other integrins, the CD11/CD18 complex seems to have originated early in evolution, as *Candida albicans* has a primitive CD18-like structure on its surface, which interacts with MAbs to the ß subunit of integrins and rosettes with C3bi-coated red blood cells, suggesting a similar epitope structure to complement receptors (Van Strijp et al., 1993; Arnaout, 1990; Hostetter, 1994).

Ligands of the Leu-CAMs bind primarily to the I domain of the receptor and seem to interact in a cation-dependent fashion, which may serve to alter the integrin binding site conformationally, or to regulate activation of the receptor (Loftus et al., 1994). C3bi, a 175kDa, active fragment of complement component C3b, is the best-studied ligand and is often used as a marker for the activation state of the α Mß2 integrin. C3bi, although containing an RGD sequence, interacts with an epitope in the I domain, but is not blocked by the use of a synthetic RGD peptide, suggesting that there is greater involvement of the flanking regions, and that the RGD tripeptide, in this instance, is not of importance in this interaction (Van Strijp et al., 1993).

ICAM 2 and ICAM 1 (CD54), members of the IgG superfamily, and factor X and fibrinogen all interact with the CD11/CD18 complex, and their epitopes can be traced

to the I domain, which is thought to have originated due to exon shuffling and subsequent reorganisation of the integrin (Loftus et al., 1994). The only known ligands to bind outside of the I domain in the CD11/CD18 complex are yeast, ß-glucan, and zymosan, but recent data question the validity of these findings (Ross et al., 1985; Newman et al., 1990; Szabo et al., 1995).

Results obtained from experiments using various ligands and cytoskeletal inhibitors suggest that there are two populations of CR3 on the cell membrane: one which is mobile and serves as a binding site only, and another that is immobile, but after interacting with its ligand will promote internalisation, due to its interaction with the cytoskeleton (Detmers et al., 1994; Graham and Brown, 1989; Roubey et al., 1991).

5.1.7 Integrin Participation During Inflammation:

During bacterial invasion or tissue damage, cytokines are produced together with bacterial and cellular chemotactic factors that stimulate the expression of the adhesion molecules ICAM-1, GMP140, ELAM-1, and VCAM-1 on the endothelium and α MÍ2 on the leukocyte. In this manner the leukocyte can interact with the upregulated ligands on the endothelium of the blood vessel and migrate through it to the site of infection or tissue damage. The orchestration of all the adhesion molecules will direct the cellular event and contain the tissue destruction. Regulation of integrin activation is targeted to the cytoplasmic tail and its interactions with the signalling pathways of the cell. This is beautifully illustrated by the tight regulation of expression of the ß2 integrins on resting versus activated macrophages: not only are the number and activity

of receptors altered on the cell surface upon activation, but also the ratio between the various receptors, depending on the destination of the cell.

Therefore, when a pathogen enters an immuno-competent host, the inflammatory response is usually able to contain and even eradicate the invading pathogen. Then how do intracellular pathogens, such as *Mycobacterium tuberculosis*, manage to establish such chronic, destructive diseases in many individuals?

As described in detail in Chapter 4, macrophages display a host of surface receptors with which the invading pathogen may interact, but not all of these will be to the advantage of the pathogen, in general. CR3 may be the preferred receptor targeted by the pathogen, as unlike the FcR and CR1, CR3-induced phagocytosis does not evoke the toxic respiratory burst or activate the cell, allowing the pathogen a safe entry into an intracellular niche that is relatively free of hostile cellular defence mechanisms, within which to multiply and establish an infection (O'Toole et al., 1994; Stokes, 1993; Isberg and van Nhieu, 1994). Our suggestion, therefore, is that CR3 may play a pivotal role in the fate of the *M.tb.* during its initial entry into the host cell. For this reason it was decided to clone CR3 into a non-phagocytic cell line, to enable evaluation of the direct interaction of the CR3 with the pathogen of interest, virulent *Mycobacterium tuberculosis*, without the presence of other phagocytic receptors to complicate the interaction.

Figure 5.2a: pπH3MCD11b and pπH3MCD18 plasmid maps

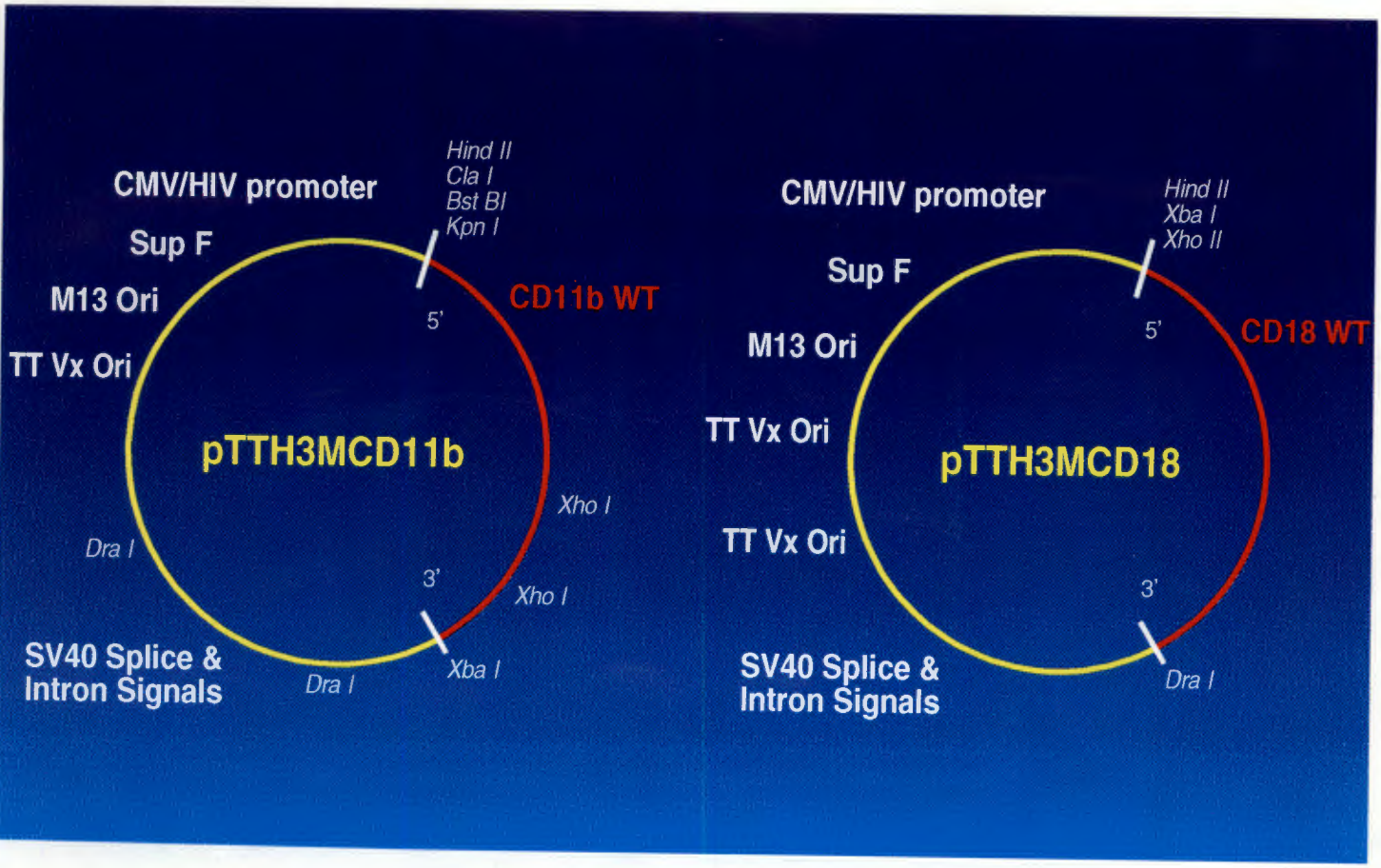
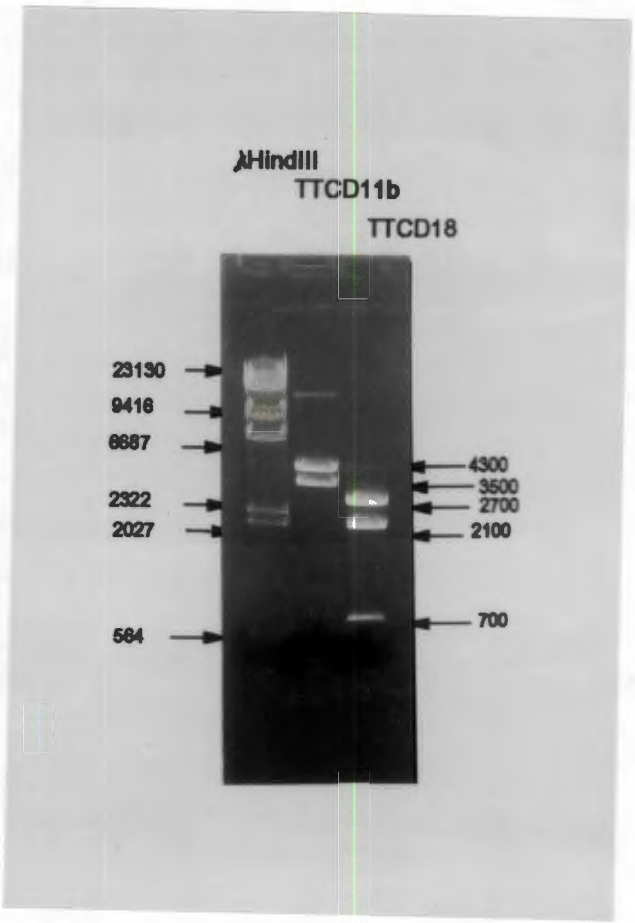


Figure 5.2b: Restriction digest patterns of pπH3MCD11b and pπH3MCD18



pπH3MCD11b and pπH3MCD18 were digested with *Hind III* and *Xba I,* and *Hinc II, Xba I* and *Dra I* respectively and the digested DNA resolved on a 0.8% agarose gel, to provide digestion fragments of 4300bp and 3500bp for pπH3M and CD11b respectively, and 2100bp, 702bp and 61bp,for pπH3M and a 2700bp fragment for CD18.

5.2 CLONING AND TRANSFECTION OF INTEGRIN CR3 (CD11b/CD18; MAC-1; αMβ2).

5.2.1 Materials and Methods:

Full-length human CD11b and CD18 cDNAs, as well as truncated human CD11b and CD18, were obtained from Dr MA Arnaout, Massachusetts General Hospital, Harvard Medical School, Boston (Arnaout et al., 1991). These were provided as inserts subcloned into the *HindIII* and *DraI* sites of the πH3M vector (Little et al., 1983), and referred to as πCD11b and πCD18, respectively (Figure 5.2a & b). The π vector contains (Figure 5.2a) a bacterial origin of replication, a selectable marker 5' to the inserted DNA fragment, and Simian Virus 40 (SV40) splice sites and termination signals to enable expression in mammalian cells. Isolation of plasmid DNA is described in Addenda A.1.1 and A.1.2.

To introduce the cDNAs into non-phagocytic CHO cells, a eukaryotic expression vector system was selected that would limit the number of synthesised receptors being expressed on the cell surface, as it was unknown at this stage if the transfected CHO cell could tolerate large numbers of receptors on its surface. The vector πH3M was not considered for direct transfection into CHO cells, as its transfection efficiency and copy number in CHO cells were unknown.

For this reason the pJ4Ω vector was chosen, as it is a useful eukaryotic expression vector, often used in CHO cell transfections in our laboratory with good results, but with low to moderate expression levels. pJ4Ω is pBR322-based eukaryotic expression

Figure 5.3a: Procedure for subcloning CD11b and CD18 cDNAs into pBluescript

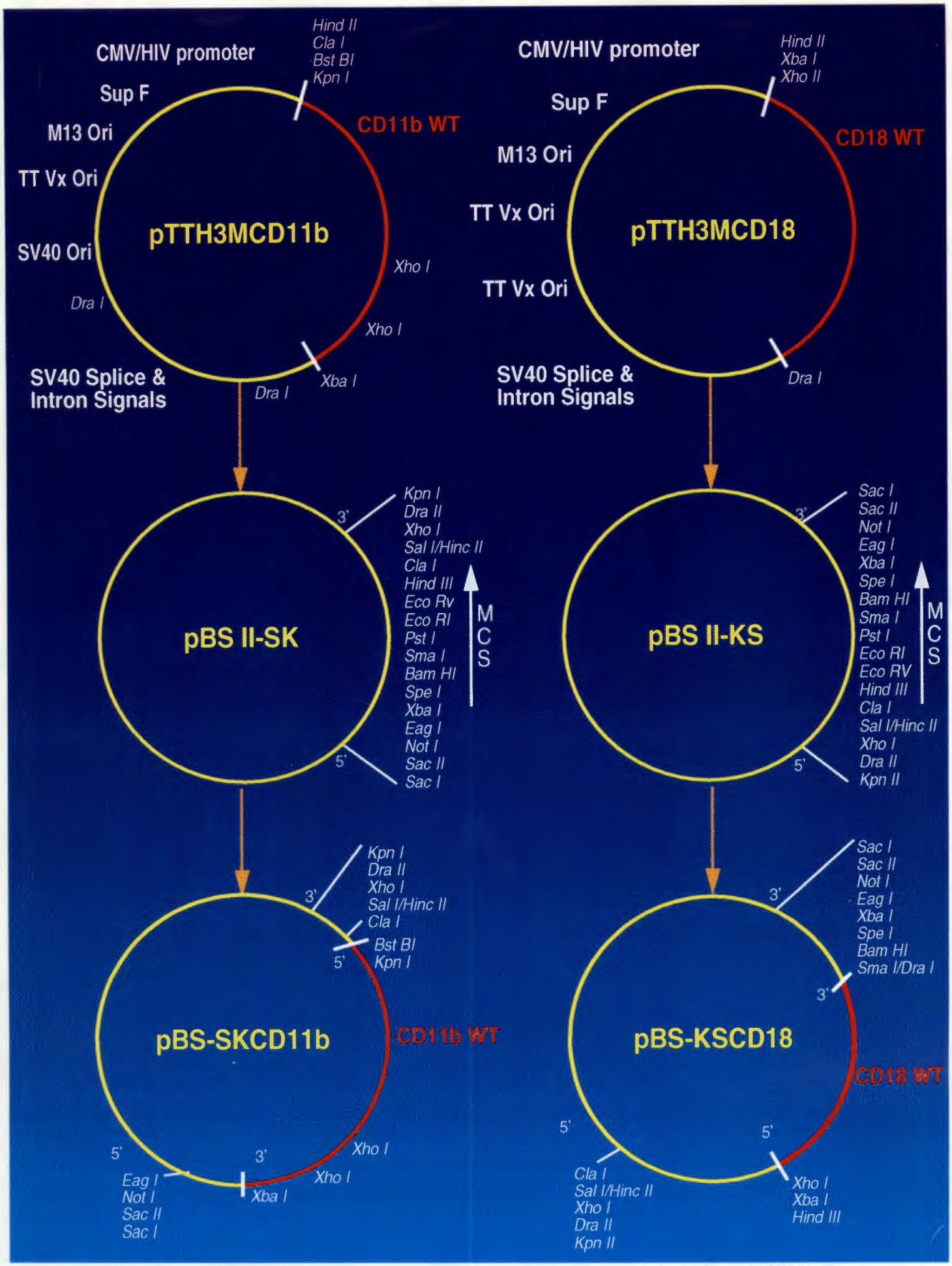
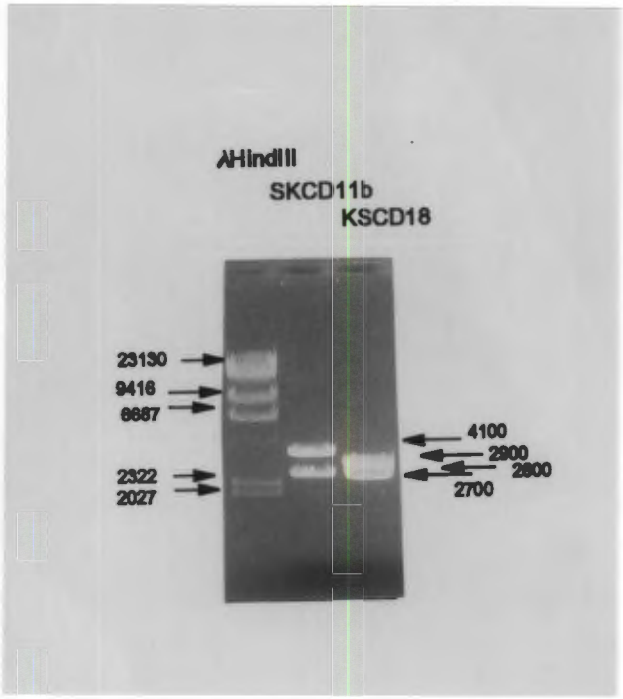


Figure 5.3b: Restriction digest patterns of pBS-SKCD11b and pBS-KSCD18



pBS-SKCD11b and pBS-KSCD18 were digested with restriction enzymes *Cla I* and *Xba I* to release the inserted cDNAs and then resolved on a 0.8% agarose gel to provide a 2900bp and a 4100bp fragment for pBS-SK and CD11b respectively, and a 2900bp and a 2700bp fragment for pBS-KS and CD18 respectively.

system (Figure 5.4a) containing the Moloney murine leukaemia virus promoter, a SV40 splice site and termination signals, which was provided in the form of pΩACE, by M.R.W. Ehlers, Medical Biochemistry, UCT (Ehlers et al., 1991).

The restriction sites available on the multiple cloning cassette of both the cDNAs and the pJ4Ω were limited, making cloning complicated, and therefore to extend the restriction sites available, initial cloning into the high copy-number *E. coli* plasmid pBluescript II (pBS) was performed. pBS, a 2961-basepair (bp) phagemid supplied by Stratagene (Figure 5.3a), originating from pUC19, is provided in two forms, pBS-SK and pBS-KS, which refers to the orientation of the multiple cloning site in relation to the lacZ transcription gene. After the pΩCD11b and pΩCD18 vectors had been constructed and CHO cells transfected, data became available from Dr T Springer's laboratory in Boston, indicating the successful transfection of CHO cells with CD11b and CD18 in a high-expression eukaryotic vector that was amplifiable, as described later. This dispelled our concerns that high surface expression levels may debilitate the cell and persuaded us to change our cloning strategy to incorporate the amplifiable expression vector pEE14. Although non-phagocytic CHO cells expressing CD11b and CD18 were available, we decided to continue our cloning strategy so as to provide us with clones expressing single subunits of either CD11b or CD18, if possible, that could be used in later studies, for further insight into the function of CR3. Similarly, the cloning of CD11b and CD18 cDNAs in which the transmembrane region had been excised to produce secreted CD11b and CD18 was continued, in the hope that it may aid the isolation of the putative *M.tb.* cell wall ligand that interacts with CR3.

Figure 5.4a: Procedure for subcloning CD11b and CD18 cDNAs into pΩACE

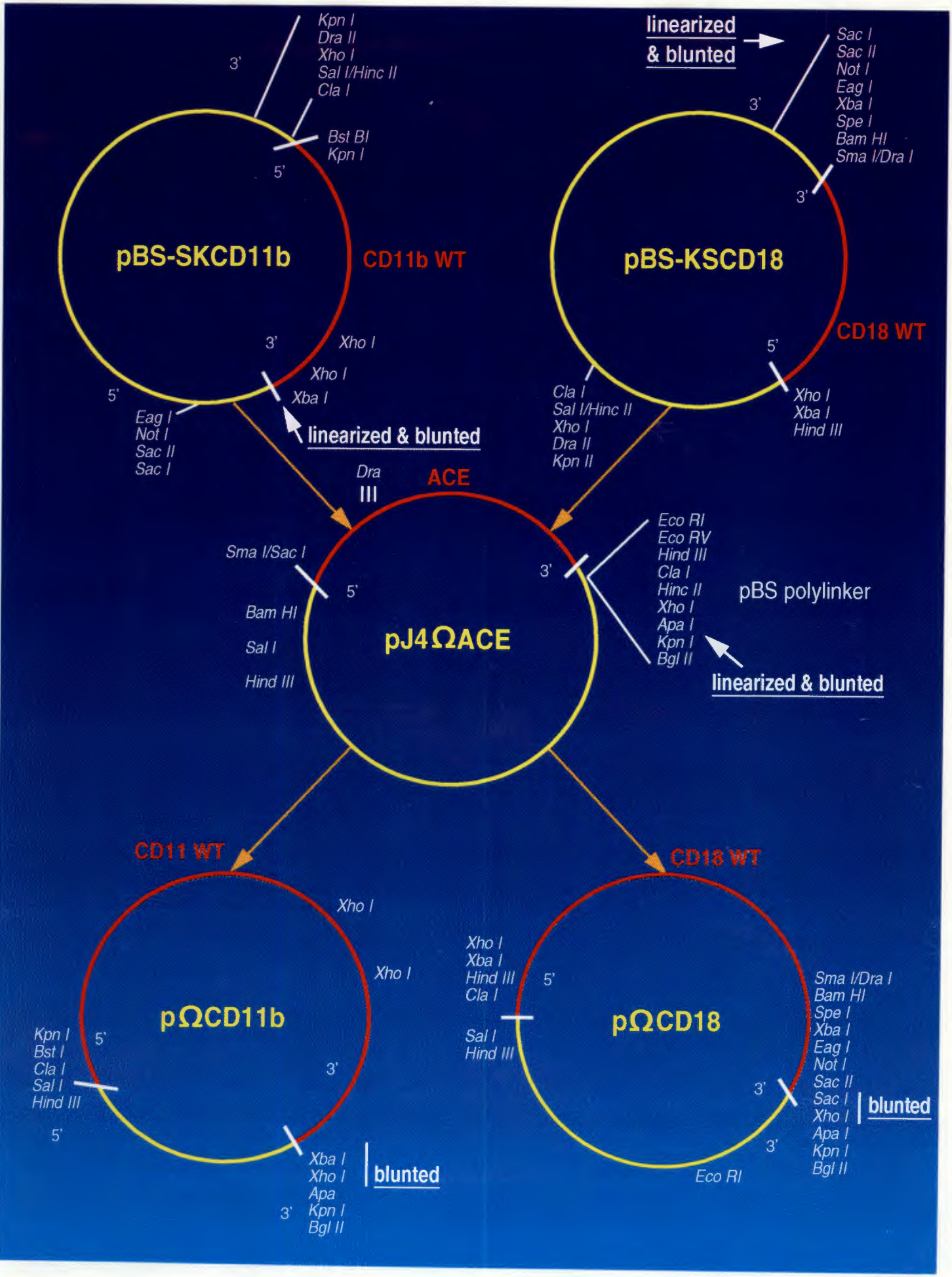
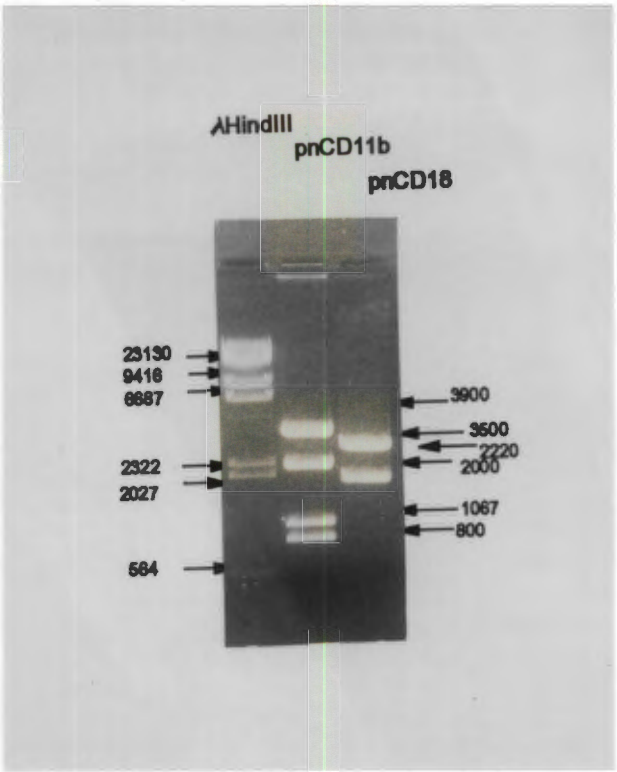


Figure 5.4b: Restriction digest patterns of pΩCD11b and pΩCD18



pΩCD11b and pΩCD18 were digested with restriction enzymes *Cla I* and *Bgl II* and resolved on a 0.8% agarose gel. Digestion fragments of 3900bp, and 2220bp, 1067bp and 800bp were released for pΩ and CD11b respectively, and 3500bp and 2100bp for pΩ and CD18 respectively.

5.2.2 Cloning Strategies:

5.2.2.1 Sub-Cloning of CD11b and CD18 cDNAs into pBluescript II:

To isolate the full-length cDNAs of CD11b and CD18, πCD11b and πCD18 were digested (experimental conditions are described in Addendum A.1.4.) with restriction enzymes *ClaI* and *XbaI*, and *HindIII* and *DraI*, respectively (Figure 5.2b). Plasmids pBS-SK and pBS-KS were similarly digested with *ClaI* and *XbaI*, and *HindIII* and *SmaI*, respectively. Digested DNA was resolved on a 0.8% low-melting-point agarose (NuSieve) gel at 50 Volts and the appropriate bands excised and purified using a DNA extraction kit (Qiaex). Purified DNA was resuspended in 10 µl of Tris/EDTA buffer (TE) and an aliquot resolved on a 0.8% agarose gel to evaluate the quantity and quality of the retrieved DNA.

The digested pBS-KS and pBS-SK were ligated to the respective CD11b and CD18 cDNA inserts, at a ratio of 1:2, vector:insert (Addendum A.1.4.). The ligation mixtures were transformed into *E. coli* strain XL-1 Blue, rendered competent by the $CaCl_2$ method (Addendum A.1.5.), and plated for selection on 100µg/ml Ampicillin Luria agar (LA) plates (Addendum A.2.). Viable colonies were selected and grown up in liquid broth to enable small-scale isolation of plasmid DNA, which was then screened by restriction mapping for correct insertion of the cDNA, Figure 5.3a. Restriction enzymes *ClaI* and *XbaI* were used to release the insert cDNA from both pBS-SKCD11b and pBS-KSCD18. All bands were electrophoresed on 0.8% agarose to verify fragment sizes (Figure 5.3b). The identity of both constructs was confirmed by nucleotide sequencing with T3 and T7 primers (Addendum A.1.8.).

5.2.2.2 Cloning of CD11b and CD18 into pJ4Ω:

Using the constructs pBS-SKCD11b (with the CD11b cDNA cloned in 3' to 5' to provide the appropriate cloning sites), pBS-KSCD18, and pΩ-ACE, the cloning strategy outlined in Figure 5.4a was followed. Essentially this involved releasing the cDNAs and cloning them into the *SalI* and *XhoI* sites of the pΩ-ACE vector, after removal of the ACE fragment, but preserving the essential features of the pJ4Ω expression vector.

To enable this, pBS-SKCD11b, pBS-KSCD18 and pΩ-ACE were linearized with restriction enzymes *XbaI*, *SacI*, and *XhoI*, respectively, heated to inactivate the enzymes, and then blunt-ended with Klenow enzyme (Addendum A.1.5.). After cleaning to remove all excess dNTPs and Klenow enzyme, the linearised plasmids were further digested with *SalI* to provide the second ligation site, releasing the CD11b and CD18 cDNAs inserts from pBS-SKCD11b and pBS-KSCD18, respectively, and the ACE cDNA from pΩ-ACE. Samples were loaded onto 0.7% NuSieve gels and resolved at 50V. After gel extraction, the CD11b and CD18 cDNAs were ligated to pΩ, at a ratio of 1:4 vector:insert. XL-1 Blue competent cells were transformed as usual and selected on Ampicillin 100µg/ml LA plates. Plasmid preparations of selected colonies grown in broth culture were digested with *ClaI* and *BglII* for both constructs, to release the appropriate fragments as indicated in Figure 5.4b. Plasmids representing the correct constructs were prepared in bulk (µg scale) by the Qiagen column purification method, as described in the manufacturer's protocol.

5.2.2.3 Transfections of CHO-K1 Cells with pΩCD11b and pΩCD18 Constructs:

Large scale (μg scale) DNA preparations of the pΩCD11b and pΩCD18 constructs, as well as the plasmid pSV2Neo, were obtained from 500ml broth cultures by the alkaline-lysis method (Addendum A.1.1), and the plasmid DNA purified by caesium chloride (CsCl) density centrifugation to obtain high quality DNA in good quantities (Addendum A.1.3.).

pSV2Neo was prepared for co-transfection with the relevant constructs, to allow selection by means of resistance to the toxic antibiotic G418 (a neomycin analogue). The pSV2Neo inserts randomly into the chromosome of the CHO-K1 cells, as do the expression vectors containing the cDNAs, thus conferring G418-resistance to the cell and enabling selection. This does not exclude the possibility that pSV2Neo integrates on its own, thereby conferring resistance to G418 without expression of the desired gene.

All CsCl-prepared constructs were dialysed to remove contaminating caesium and salts and the quantity and quality of the DNA estimated by absorption at OD_{260} and OD_{280}. In all cases, OD_{260}/OD_{280} ratios exceeded 1.8, and DNA concentrations were estimated:

SV2Neo = 5.25 μg/ml; pΩCD11b = 19.8 μg/ml; and pΩCD18 = 39.8 μg/ml.

A Chinese hamster ovary (CHO) fibroblast cell line (CHO-K1; ATCC CCl 61) was grown in complete media (Addendum A.2.) to 75% confluence in Nunc tissue culture flasks, after which the cells were lifted with trypsin/EDTA, washed, reseeded at

approximately 1 x 10^6 cells/100mm petri dish in complete media, and left overnight at 37°C with 5% CO_2.

Three hours prior to transfection, the cells were washed and the media replaced. The cells were co-transfected with either pΩCD11b and pSV2Neo, pΩCD18 and pSV2Neo, or triple-transfected with pΩCD11b, pΩCD18 and pSV2Neo, using the calcium phosphate ($CaPO_4$) precipitate method, as described in detail in Addendum A.1.10. The precipitate was left on the cells for 4 hours at 37°C with 5% CO_2, after which the cells were gycerol-shocked, to enhance DNA uptake, for two minutes at 37°C with 5% CO_2. The glycerol was removed by repeated washing, the cells refed with complete media, and grown at 37°C with 5% CO_2 until confluent. At confluence, the cells were split into 48 well plates and allowed to adhere overnight in complete media at 37°C with 5% CO_2. The following day the cells were washed and refed with complete media containing 0.4 mg/ml of G418 to initiate the selection of resistant clones. As colonies started appearing, they were lifted and seeded into small 25ml flasks to obtain clones. Resistant colonies were screened for expression of CD11b/CD18 by Fluorescence Activated Cell Sorting (FACS).

5.2.2.4 FACS Screening of CD11b- and CD18-Expressing CHO Cell Clones:

Fluorescein isothiocyanate (FITC)- or R. Phycoerythrin (PE)-conjugated MAbs to either the CD11b or the CD18 subunit were used, to determine cell surface expression of the respective subunits by dual-colour flow cytometry. Antibodies used were as follows. From Serotec were purchased mouse anti-human CD11b, FITC-conjugated MAb (clone 44), rat anti-human CD18, RPE-conjugated MAb (clone YFC118.3), and

FITC-labelled mouse IgG1, as an isotypic control for the mouse anti-human CD11b; no isotypic control was available for the rat MAb. Second, from Dako were purchased mouse anti-human CD11b, RPE-conjugated MAb (clone 2LMP19c), mouse anti-human CD18, FITC-conjugated MAb (clone MHM23), and dual-colour isotypic control mouse IgG1, FITC- and RPE-conjugated. Untransfected CHO-K1 cells (designated CHO-WT) and CHO-MAC-1 cells (see below) were used as negative and positive controls, respectively, in all experiments.

Stably transfected cells and controls were grown to 75% confluence, lifted in 5mM EDTA/HBSS (without trypsin to preserve the receptor), and resuspended at $\pm 2 \times 10^6$ cells/100µl in 1:100 dilution of MAb or isotypic control in PBS. Cells were incubated on ice for 15 minutes, after which 500 µl of 1M paraformaldehyde was added to crosslink the receptors. Cells were kept at 4°C for a maximum of 48 hours before screening.

Results from the first round of FACS screening were disappointing, with very low levels of receptor expression; this was not improved after re-selection with G418. An attempt was then made to enrich for CD11b- and CD18-expressing cells, by immunopanning.

This procedure was performed as follows. Plastic petri dishes (non-tissue culture treated) were coated with 50 µg/ml of Rabbit-anti mouse-Ig (Dako) in PBS for 40 minutes at room temperature, with continual swirling. CHO cells were grown in complete media, lifted with 5mM EDTA/HBSS, and washed to remove all traces of EDTA. The cells were incubated with anti-CD11b and anti-CD18 MAbs, clones 2LPM19c and MHM23, respectively (Dako), for 20 minutes at room temperature, shaking. During this time, the coated petri dishes were washed extensively with PBS

containing 1% FCS (PBS-FCS), to block any non-specific binding. After washing the cells with PBS-FCS, to remove excess MAb, the cells were resuspended in 3ml of PBS-FCS and poured onto the coated plates and allowed to adhere for 20 minutes at room temperature, shaking gently. The non-adherent cells were decanted and the adherent cells washed five times with PBS-FCS. The adherent cells were trypsinised off the coated petri dish and re-plated and incubated in complete media at 37°C with 5% CO_2, to adhere (Diamond et al., 1990). Each clone was grown to confluence in selective complete media and repanned to optimise selection.

Cell surface receptor expression was re-evaluated for each clone, with FACS, as discussed in the results. These results were disappointing and suggested that the expression of the transfected receptors, was transient. As discussed previously, the cloning strategy was diverted and the CD11b and CD18 cDNAs cloned into pEE14, for amplifiable expression.

5.2.2.5 MAC-1 Springer Cells:

While the construction of pΩ-CD11b and pΩ-CD18 were in progress, Dr TA Springer, Centre for Blood Research, Harvard Medical School, Boston, reported the stable expression of CR3 in CHO cells, in a cell-line designated CHO-MAC-1. Selection and stable expression in these cells was based on the dhfr minigene in the pDCHIP plasmid, which was co-transfected with pCDM8 containing human, wild-type CD11b or CD18 cDNA into CHO DG44 cells. CHO DG44 cells have the dihydrofolate reductase gene deleted. Transfected clones were selected in nucleoside- and nucleotide-deficient media (Alpha-MEM with dialysed FCS), and amplified with

methotrexate (MTX), a dihydrofolate reductase inhibitor (Addendum A.2). CR3-expressing cells were enriched by immunopanning and screened by FACS (Diamond et al., 1990; Current Protocols in Molecular Biology, 1991; Bebbington and Hentschel, 1987).

From these results it was evident that high levels of heterologous CR3 expression in CHO cells could be attained, which ruled out our initial concerns that led to our use of pJ4Ω as the vector of choice. While we continued with our own transfections, we acquired the CHO-MAC-1 cells from Dr Springer as positive controls for our experiments.

5.2.2.6 Cloning of CD11b and CD18 into pEE14:

Based on the success Springer reported with MTX-amplified expression of CR3 in CHO cells, we constructed additional expression plasmids for CD11b and CD18, using the amplifiable pEE14 vector, which is based on the glutamine synthetase minigene. The pEE14 vector contains an hCMV-MIE promoter-enhancer, an SV40 late promoter, and the glutamine synthetase minigene as the selection/amplification marker (Bebbington and Hentschel, 1987) (Figure 5.5a). pEE14 was obtained from Celltech. The CD11b and CD18 cDNAs were subcloned into pEE14, to generate pEECD11b and pEECD18, respectively, as outlined in Figure 5.5.

The πCD11b and πCD18 vectors were digested with restriction enzymes *HindIII* and *XbaI*, and *XbaI* and *DraI*, respectively; pEE14 was digested with *HindIII* and *XbaI*, and *HindIII* and *SmaI* for CD11b and CD18, respectively (Figure 5.5a). *SspI* and *HincII* were used as additional enzymes to cleave the π vector of πCD18 into smaller

Figure 5.5a: Procedure for cloning CD11b and CD18 cDNAs into pEE14

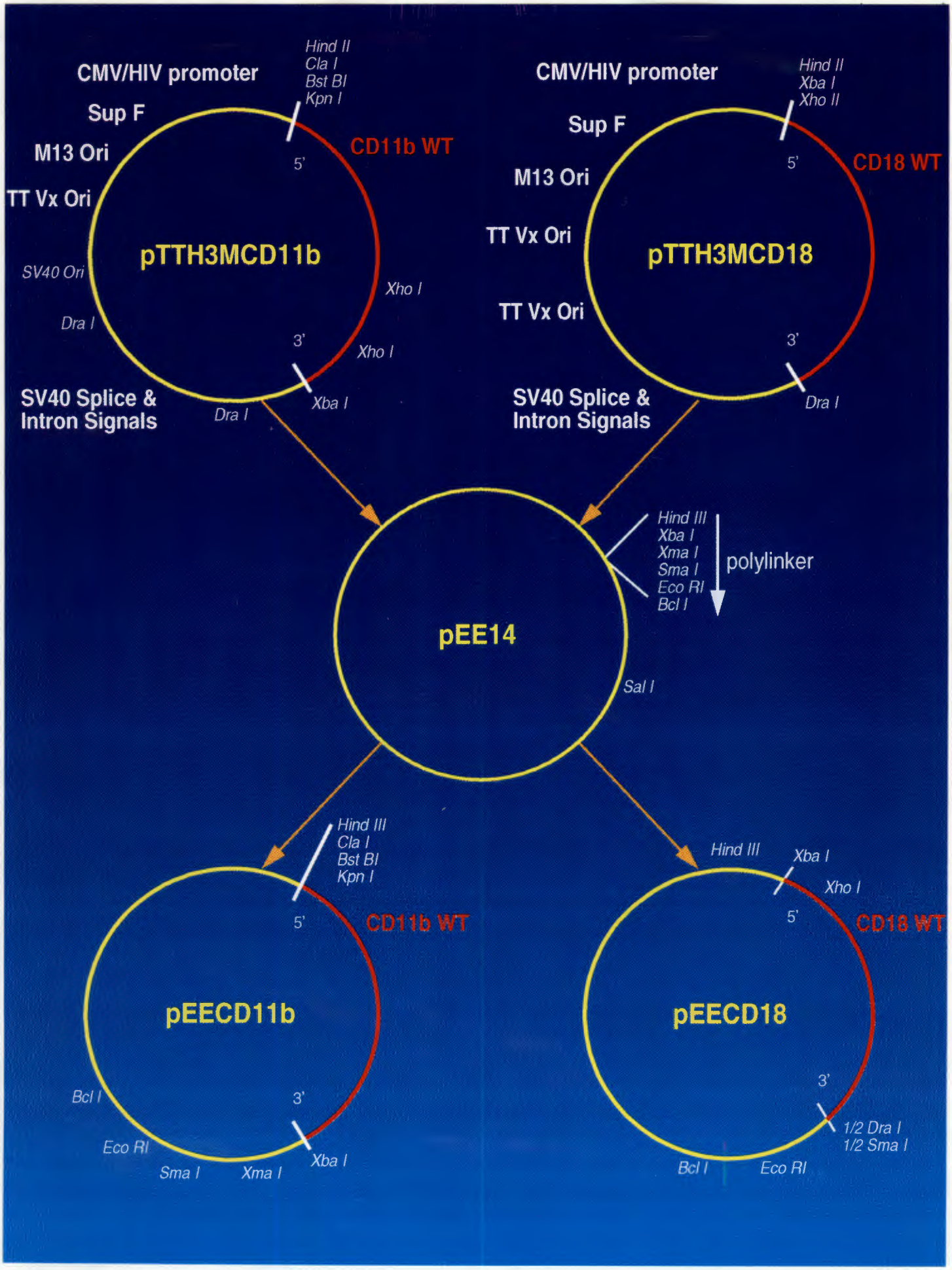
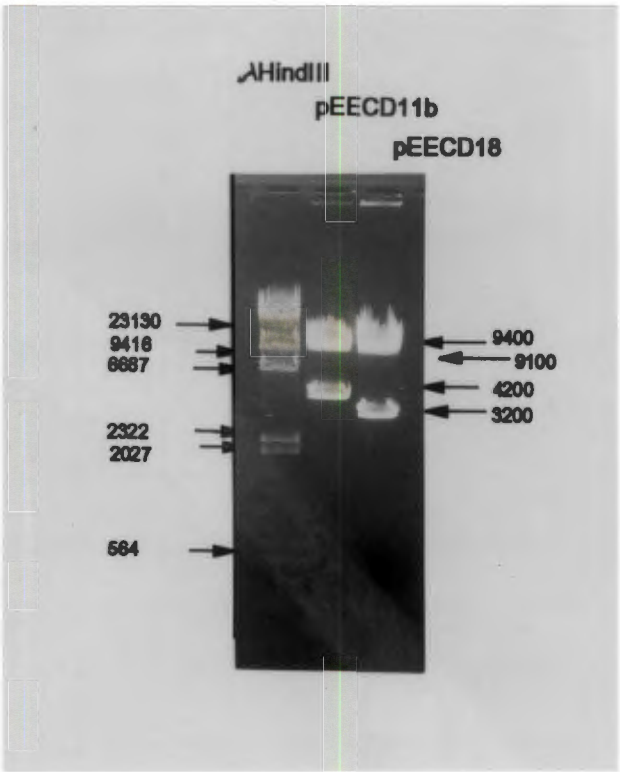


Figure 5.5b: Restriction digest patterns of pEECD11b and pEECD18



pEECD11b and pEECD18 were digested with *Hind III* and *Xba I* (pEECD11b), and *Xba I* and *Sal I* (pEECD18) and resolved on a 0.8% agarose gel. Fragment sizes of 9400bp and 4200bp were ressolved for pEE and CD11b respectively, and 9100bp and 3200bp for pEE and CD18 respectively.

fragments, facilitating their electrophoretic separation from the CD18 insert (Figure 5.5b). The appropriate bands were excised and extracted from a 0.8% NuSieve gel and cleaned prior to ligation. The ligations were performed as described previously at a ratio of 1:2 vector:insert (Addendum A.1.4), and XL-1 Blue cells transformed (Addendum A.1.6) and selected on Ampicillin 100 μg/ml. Colonies were screened by digesting small-scale plasmid preparation DNA with *HindIII* and *XbaI* (pEECD11b) and *XbaI* and *SalI* (pEECD18) to release the insert cDNAs (Figure 5.5b).

Constructs identified as correct were grown up for large-scale preparations and purified by CsCl gradient centrifugation (Addendum A.1.3.). The DNA was assessed by absorption at OD_{260} and OD_{280}. Concentrations were found to be 1 μg/ml for pEECD11b and 1.6 μg/ml for pEECD18.

5.2.2.7 Transfection of CHO-K1 Cells with pEECD11b and pEECD18:

Transfection conditions for pEECD11b and pEECD18 were similar to those described for the pΩ constructs. An important difference was the use of glutamine-deficient media (Addendum A.2.), which is required for the selection procedure. CHO cells were grown in the GMEM complete media to adapt the cells, before transfection with the pEE constructs. The cells were transfected as with the pΩ constructs, but without the pSV2Neo vector. Following transfection and after the cells had reached confluence, Methionine sulphoximine (MSX) was added to the media. Selection was initiated at a concentration of 25 μM MSX in complete GMEM, and the surviving clones grown to confluence. Gene expression was amplified by increasing the MSX concentration to a final concentration of 300 μM MSX, in 50 μM steps.

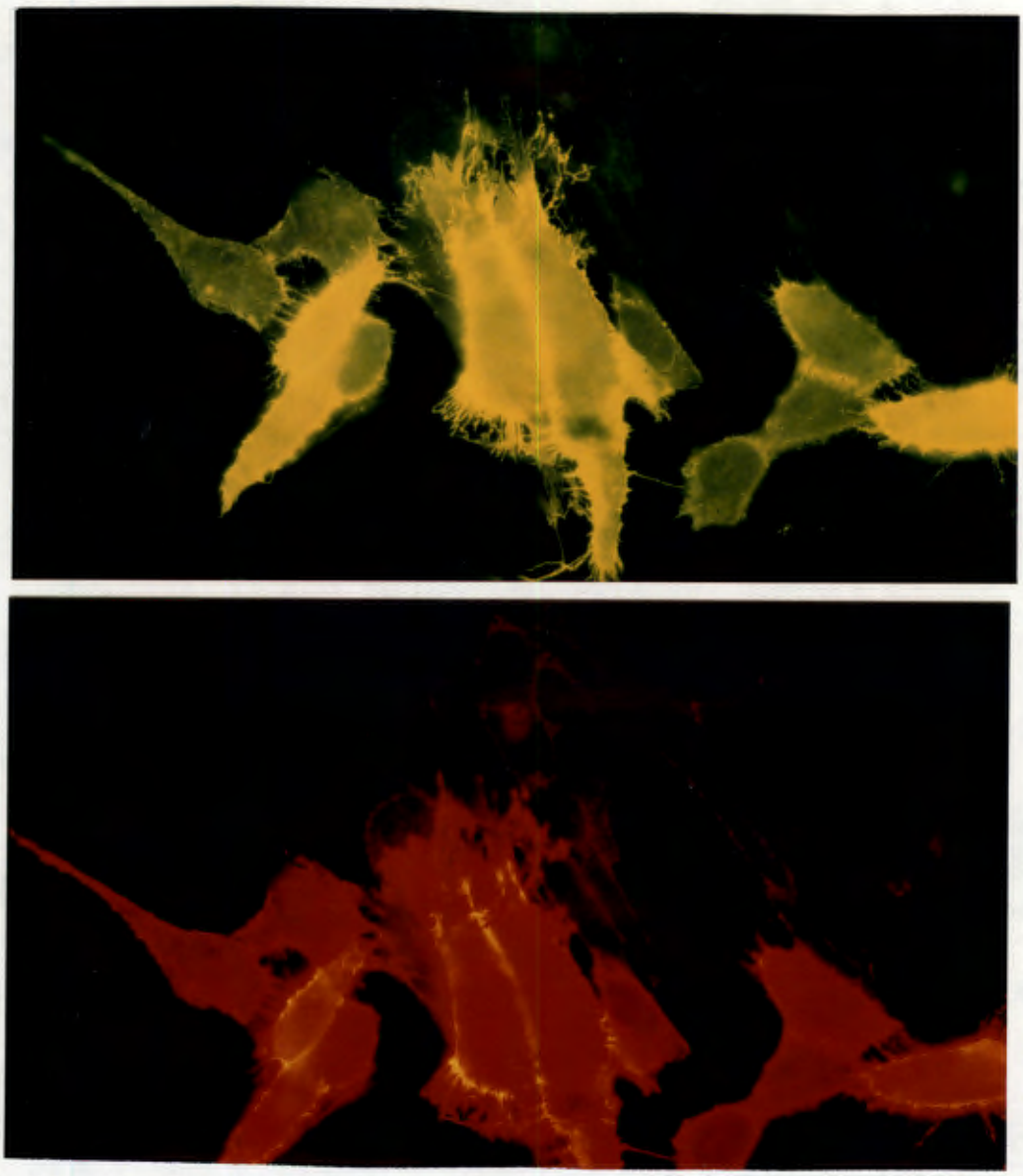
5.2.2.8 Surface Expression of CD11b and CD18 in MSX-Resistant CHO Cell Clones:

Cells were grown to confluence in complete GMEM media containing 100 μM MSX, and plated on 16mm glass coverslips at a density of 1 x 10^5 cells/coverslip. Surface expression of CD11b and CD18 was then determined by one of two methods.

Indirect immunofluorescence: This was used to assess the percentage of cells expressing CD11b/CD18. Cells grown on the coverslip were air dried and fixed in methanol for 1 minute at room temperature. The cells were blocked in 5% skim milk/Tris-Buffered Saline (S-TBS) for 30 minutes at room temperature, after which 490 μl of fresh S-TBS containing 10 μl MAb, mouse anti-human CD11b or anti-CD18 (1:50 dilution), was added and the cells incubated for 30 minutes at 4°C. The cells were washed and a 1:50 dilution in S-TBS of FITC-conjugated anti-mouse Ig was added for 30 minutes at 4°C. Cells were washed extensively in PBS to remove all unbound label, and the coverslips were inverted onto glass slides and fixed with nail polish before examination by fluorescence microscopy (Figure 5.6).

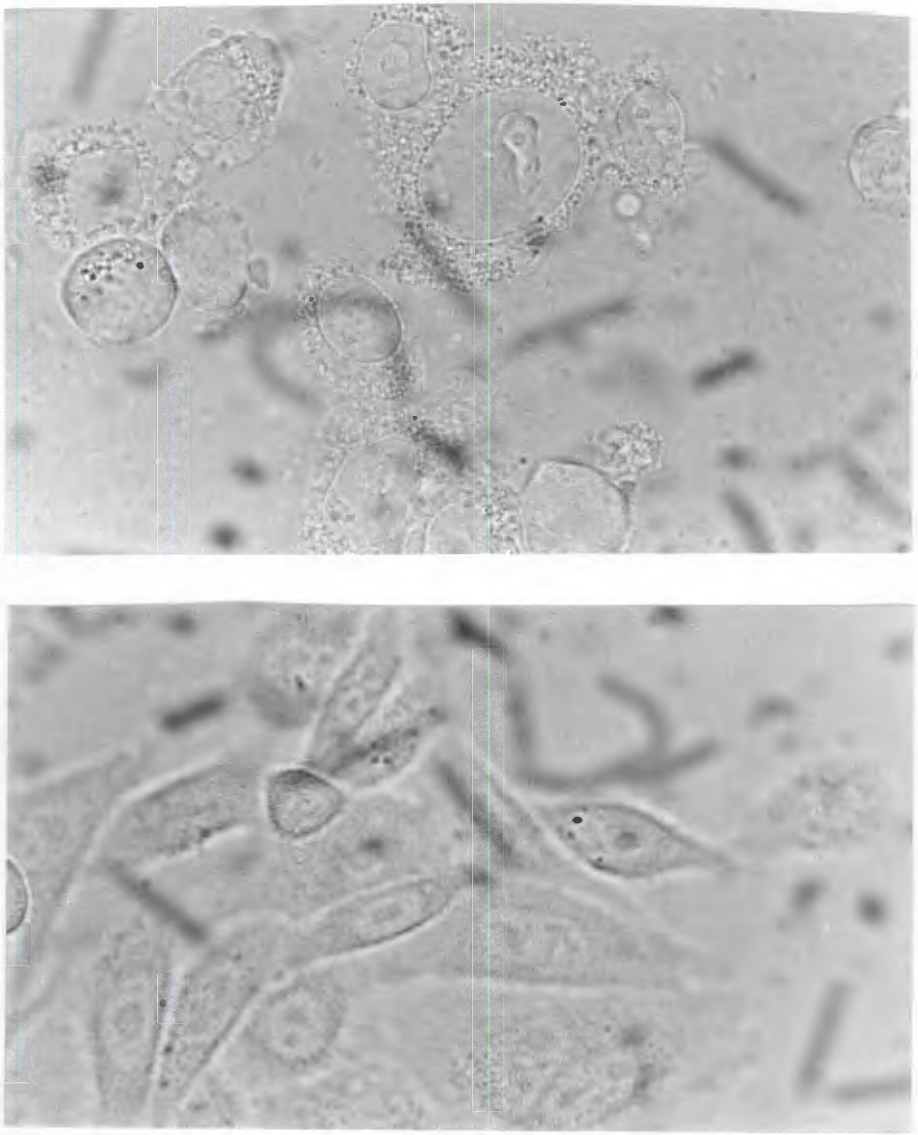
Subsequently, we also used direct immunofluorescence. In this procedure, cells were grown overnight on 12mm glass coverslips as before and then washed well with PBS, to remove all media. The cells were incubated with 10 μl of either FITC-labelled mouse anti-human CD18 or RPE-labelled mouse anti-human CD11b (Dako) in 100 μl of 1% BSA, for 1 hour at 4°C. The cells were then washed gently with PBS three times and once with 1% BSA, and fixed with 1% PFA. Coverslips were lifted and sealed as described above, before microscopy.

Figure 5.6: Immunofluorescent staining of pEECD11b- and pEECD18-transfected CHO cells



Cells grown overnight on glass coverslips were incubated with either (A) FITC-labelled CD18 or (B) RPE-labelled CD11b for 1 hour at 4°C, washed and fixed with PFA, before viewing with fluorescent microscopy.

Figure 5.7: Surface expression of CD11b/CD18 as determined by immunobead rosetting



Cells grown on glass coverslips were incubated with CD11b/CD18 MAb-treated beads for 30 minutes at room temperature, before viewing with light microscopy. (A) represents cells expressing both CD11b and CD18 and (B) represents the control CHO-WT cells which do not express CD11b or CD18.

Immunobead assay: This was used to obtain an additional semi-quantitative estimate of CD11b/CD18 surface expression on transfected cells. In this method, protein A-agarose beads were coated with either mouse anti-human CD11b or anti-human CD18 MAb. Conjugation with protein A-agarose beads was achieved by incubation with 1 part MAb and shaking for 1 hour at room temperature. The beads were washed with 10 volumes of 0.2M sodium borate ($Na_2B_4O_7$), pH9.0, and pelleted at 3000 x g for 5 minutes. The beads were resuspended in 10 volumes of 0.2M sodium borate , pH9.0, containing dimethylpimelimidate (DMPD) (Pierce) at a final concentration of 20 mM. After 30 minutes of shaking at room temperature, the reaction was stopped by washing the beads in 10 volumes of 0.2M glycine, pH 8.0, and incubating in the same buffer for 2 hours at room temperature. The beads were finally washed with PBS and stored in 10 ml PBS with 0.02% sodium azide at 4°C. Rosetting of the beads with cells expressing CD11b/CD18 was determined after incubating cells grown on coverslips with the MAb-treated beads for 30 minutes at room temperature. The cells were scored by light microscopy, as indicated in Figure 5.7 (Sigal et al., 1994).

5.2.2.9 Functional Activity of CR3 Expressed in Transfected CHO Cells:

One of the best characterised ligands for CR3 is complement component C3bi, which binds to the I domain of CD11b. The functional activity of the recombinant receptor was assessed by the binding of C3bi-coated microspheres. The latter were generated by first coating the microspheres with phenolic glycolipid (PGL-1) of *M.leprae*, which when incubated with fresh serum activate the classical complement pathway, leading to the deposition of C3bi on the surface of the microspheres (Schlesinger and Horwitz,

1991). 1μM-diameter polystyrene microspheres were washed in a 0.05M carbonate-bicarbonate buffer, pH 9.6, (CB-buffer), and then incubated with 100 μg of PGL-1 (a kind gift from Patrick Brennan, Colorado State University, Fort Collins, Colorado) in CB-buffer for 2 hours at 37°C, shaking. Control microspheres were incubated with CB-buffer alone. Both sets of beads were washed with PBS and blocked by incubating in PBS containing 5% bovine serum albumin (BSA), for 2 hours at 37°C, shaking. Beads were washed again and incubated in PBS containing 0.5% BSA and reincubated for 30 minutes at 37°C shaking in 500 μl 2.5% fresh human serum. The microspheres were thoroughly washed and resuspended in 500 μl 0.5% BSA PBS (Schlesinger and Horwitz, 1991).

To confirm that serum opsonization of PGL-1-coated microspheres results in the fixation of C3 on the particle surface, we incubated opsonized particles with FITC-conjugated anti-human C3c antibody and analysed the immunofluorescence by flow cytometry (Figure 5.8a). Opsonized PGL-1_coated microspheres were 95.6% positive for staining with anti-C3c-FITC compared to 4.9% of unopsonized PGL-1-coated microspheres (Figure 5.8b).

Both sets of beads were incubated with the CR3-expressing cells and CHO-WT cells, grown on glass coverslips, for 24 hours in serum-free DMEM/F12 (incubation media), at 37°C, with 5% CO_2. Cells were washed repeatedly to remove the non-adherent microspheres, cross-linked with 2.5% glutaraldehyde for 1 hour at 4°C, and then briefly counter stained with Trypan blue, for contrast. The cells were examined under light microscopy using a 100x objective, and the results were quantitated by estimating the percentage of cells that bound one or more microspheres, as well as the number of

microspheres bound per cell. Approximately 100-200 cells were counted per coverslip (Figure 5.9).

5.2.2.10 Transient Transfections of pJ4Ω and pEE14 Clones:

Because of initial difficulties in obtaining stably transfected cells, we also established transient transfections of CD11b and CD18 in CHO cells. The transient transfections were based on the plasmids already described, namely pΩCD11b, pΩCD18, pEECD11b, and pEECD18. The transfection methods were identical to those used for stable transfections, but selection with either G418 or MSX was omitted. Instead, the cells were used within 72 hours of transfection. Receptor expression in these clones was verified by means of indirect or direct immuno-fluorescence before use in in vitro assays.

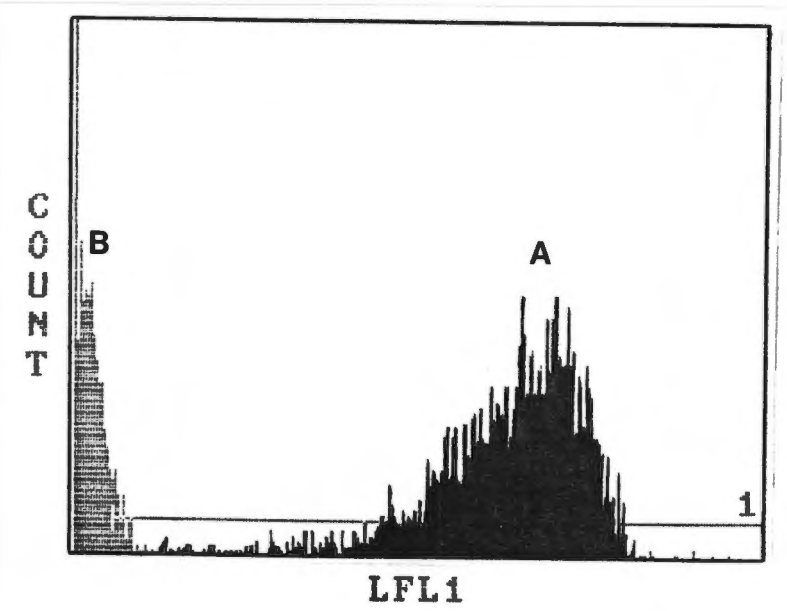
5.3 CLONING AND TRANSFECTION RESULTS:

5.3.1 Expression of CR3:

5.3.1.1 FACS Results of pJ4Ω Clones:

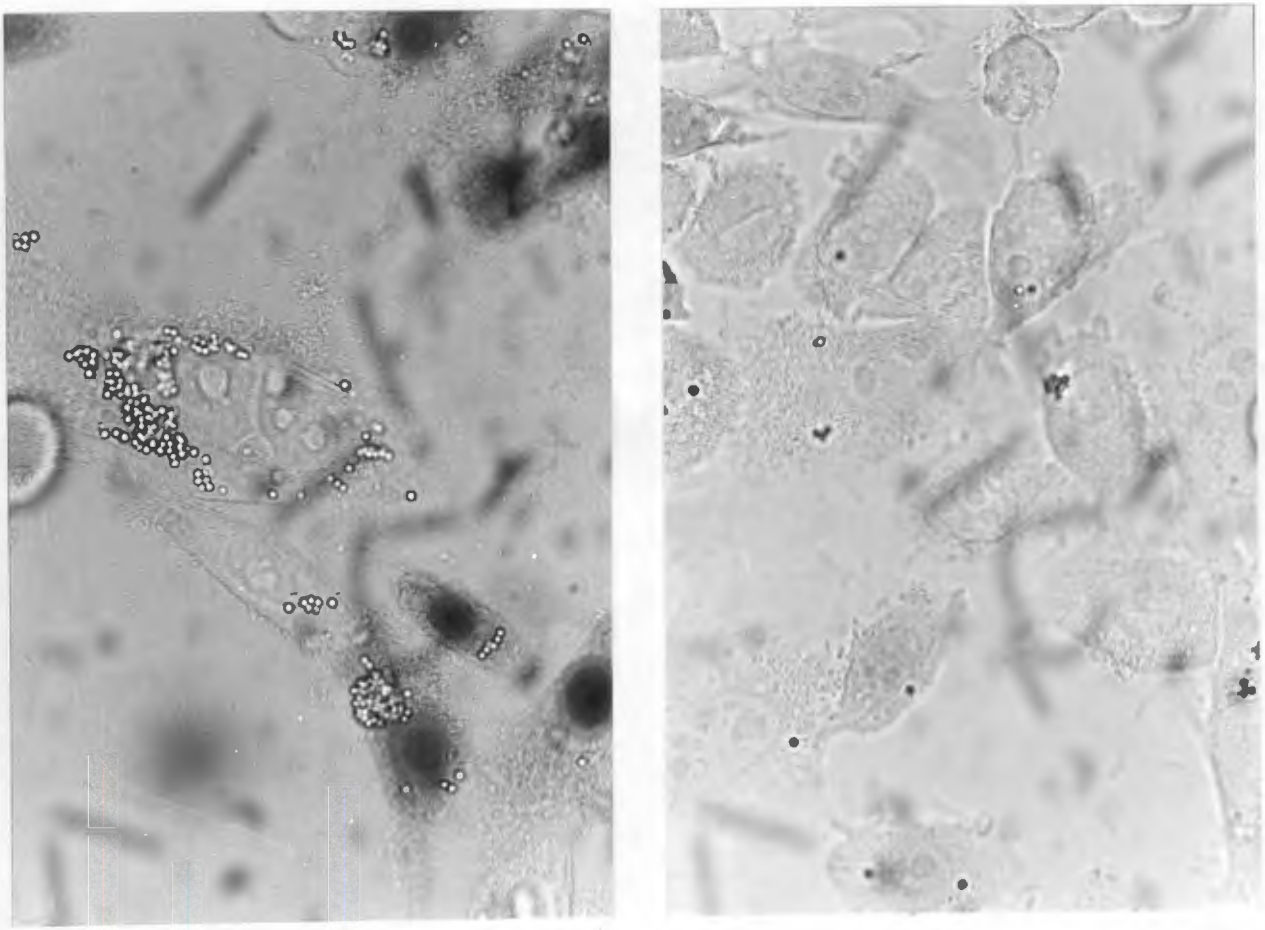
FACS was used to identify the number of transfected CHO cells expressing CD11b, CD18, or both CD11b and CD18, on their cell surface. The pΩ-transfected CHO cells were incubated with either FITC-labelled anti-CD11b MAb or RPE-labelled anti-CD18

Figure 5.8: Flow cytometry results of C3bi-coated microspheres



Microspheres were coated with PGL-1 and opsonized with fresh human serum. To confirm the opsonization, beads were incubated with FITC-conjugated anti-human C3c antibody and analysed by flow cytometry (A). Similarly, beads coated only with PGL-1 were used as controls (B).

Figure 5.9: Binding of C3bi-coated microspheres to CD11b/CD18-expressing CHO cells



Cells grown overnight on glass coverslips were incubated with (A) microsphere coated with PGL-1 and fresh serum or (B) with microspheres only incubated in fresh serum to indicate the functional activity of the CR3 expressed in CHO cells, with regard to binding of CR3 ligand C3bi.

MAb for the single transfected cells, and dual labelling with both MAbs for the co-transfected CHO cells. CHO-WT and CHO-MAC-1 cells were used as negative and positive controls, respectively.

Three weeks after selecting the pΩ transfected clones for resistance to G418, the cells were lifted off the tissue culture dishes and incubated with the above fluorescent-labelled MAbs and screened with FACS. Dead cells were gated out using forward and side light scatter parameters. Of the 2000-6000 cells screened in this manner for each transfection, only 4-18% of the CD11b-transfected CHO cells, 44-55% of the CD18-transfected CHO cells, and 2-31% of the dually transfected CHO cells were positive, as compared to the negative control CHO-WT cells and the relevant isotypic controls (Table 5.3). Due to the low number of cells expressing the various transfected receptors, immunopanning was used to attempt to enrich the cell population for those expressing the required receptor only.

Once the immunopanned cells had increased in number, they were rescreened by FACS, to assess whether enhancement of receptor expression had been achieved. These results indicated two cell populations of differing cell size in each of the transfected cell lines, suggesting a mixed cell population, but both populations of cells evidenced enhanced receptor expression. As indicated in Table 5.4a, CD11b expression was enhanced to 18% and 87%, CD18 expression to 7.2% and 18%, and CD11b/CD18 dual expression to 54% and 64%, for each of the subpopulations, as compared to the positive control, CHO-MAC-1 cells, and negative control, CHO-WT cells (Figure 5.10).

Table 5.3: FACS Analysis of pΩCD11b/CD18-Transfected CHO Cells

SUBUNIT	CLONE NUMBER	% EXPRESSION
CD11b	9	5
	15	18
	38	12
	41	8
	46	4
CD18	26	47
	31	55
	37	44
	38	48
CD11b/CD18	8	16
	9	2
	24	12
	25	31
	27	27
	36	20

Cells were grown to 75% confluence, lifted in 5 mM EDTA/HBSS and resuspended at ±2 x 10_6 cells/100µl in 1:100 dilution of MAb. Cells were incubated on ice for 15 minutes, after which 500µl of 1M paraformaldehyde was added to each sample. Samples were kept at 4°C until screening.

Due to the relatively good enrichment of receptor expression by immunopanning, a second round of panning was performed to purify the cell population further. At this stage the transfected cells were 9 weeks post transfection. The repanned cells were prepared for FACS as before. Both populations of cell were again visible in each of the transfected cell clones. The expression of these repanned cells had decreased substantially to 1.5% and 2.2% for the CD11b transfectants, 1.9% and 1.9% for the CD18 cells, and 5.0% and 3.8% for the dual transfectants, for each subpopulation. These results were not much above background expression as indicated in Table 5.4b, suggesting transient expression of the transfected receptors. Subsequent cloning was pursued with the pEE14 vector, as described.

5.3.1.2 Immunofluorescence:

Using a combination of anti-CD11b and anti-CD18 MAbs, CHO cells transfected with both pEECD11b and pEECD18, either transiently (CHO-CR3T) or stably (CHO-CR3S), and CHO-MAC-1 cells expressed fluorescence. Approximately 60% of the cells were moderately fluorescent, with 10% of the cells fluorescing brightly. In comparison, the CHO-WT cells fluoresced only weakly or not at all (Figure 5.6). When using direct immunofluorescence, 65% of all the cells were fluorescent, with relatively similar amounts of CD11b and CD18 fluorescing.

5.3.1.3 Cell-Bead Immunoassay:

Protein A beads coated with a MAb to CD11b (2LPM19c) rosetted with 92% of CHO-MAC-1 cells, 97% of CHO-CR3S, and only 12% of CHO-WT cells. Similar results were obtained with beads coated with an anti-CD18 MAb (MHM23), in that

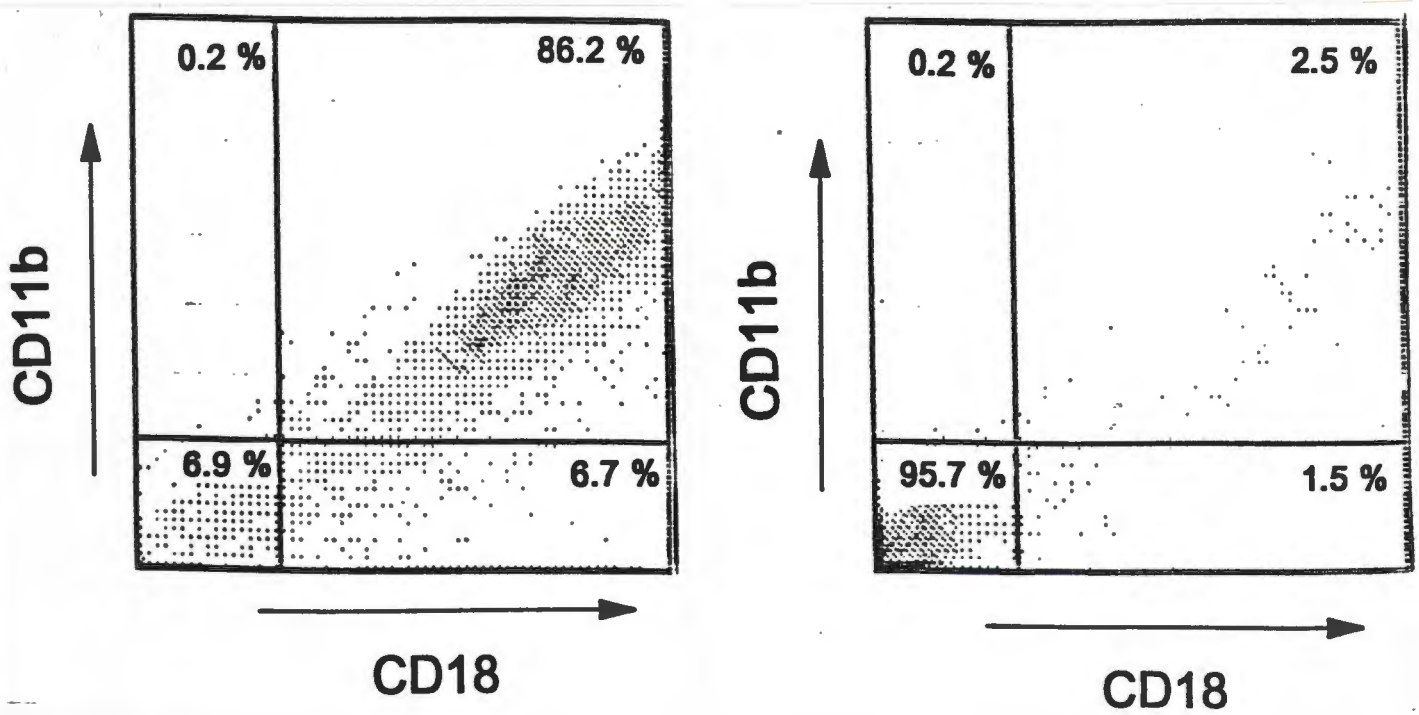
Table 5.4a: FACS Analysis of pΩCD11b/CD18 Clones after First Immuno-panning

SUBUNIT	CLONE NUMBER	% EXPRESSION POPULATION 1	% EXPRESSION POPULATION 2
CD11b	15	18	87
CD18	31	7	18
CD11b/CD18	8	50	64
	8b	30	77
CHO-WT (FITC)	-	4	
CHO-WT (PE)	-	5	
CHO-MAC-1 (Dual)		60	

Table 5.4b: FACS Analysis of pΩCD11b/CD18 Clones after Second Immuno-panning

SUBUNIT	CLONE NUMBER	% EXPRESSION POPULATION 1	% EXPRESSION POPULATION 2
CD11b	15	1.5	2.2
CD18	31	1.9	1.9
CD11b/CD18	8	5.0	3.8
CHO-MAC-1 (Dual)		53	93
CHO-WT (Dual)		2.2	1.9
(FITC)		2.2	4.4
(PE)		1.7	1.4

Figure 5.10: FACS results of CHO-MAC-1 cells



Cells were labelled with both FITC-conjugated mouse anti-human CD18 and RPE-conjugated mouse anti-human CD11b, or with similarly conjugated irrelevant mouse IgG1 as the isotypic (negative) control.

Table 5.5: Cell-immunobead Results

CELL TYPES	MAbs	
	CD11b (2LPM19c)	CD18 (MHM23)
CHO-CR3S	97 %	94 %
CHO-MAC-1	92%	90%
CHO-WT	12%	10%

Protein A beads coated with a MAb to either CD11b or CD18 were incubated with cells expressing CD11b/CD18 for 30 minutes at room temperature. The cells were scored by light microscopy as to the number of cells associated with the beads.

90%, 94%, and 10% rosetting was observed with CHO-MAC-1, CHO-CR3S, and CHO-WT, respectively (Table 5.5). These results indicate that the CHO cells transfected with pEECD11b and pEECD18 express CD11b and CD18 at levels comparable to the well documented CHO-MAC-1 cell line (Diamond et al., 1993).

5.3.2 Functional Binding Activity of CR3:

Functional activity of the recombinant CR3 expressed in CHO cells was assessed by the ability of transfected cells to bind C3bi, a well characterised ligand of CR3. For ligand binding to occur, the α and ß subunits must associate as conformationally active heterodimers, with exposure of the epitopes required for ligand binding (Arnaout, 1990). The cells transfected with the pEE constructs were compared to CHO-WT (negative control) and CHO-MAC-1 (positive control) cells. An additional negative control consisted of untreated microspheres that have no C3bi bound to their surface. C3bi-coated polystyrene microspheres and control microspheres were incubated with the cells and the percentage of cells associated with beads determined. Association of microspheres was observed in 53% of the CHO-MAC-1 cells, 76% of the CHO-CR3S and in 12% of the CHO-WT cells (Table 5.6). With the use of the control microspheres, 12% of CHO-MAC-1, 18% of CHO-CR3S and 15% of CHO-WT were associated with microspheres. These results indicate that at least a proportion of surface-expressed CD11b and CD18 subunits have associated to form active CR3 heterodimers that bind the ligand C3bi.

Table 5.6: C3bi-coated Microsphere Assay Results

CELL TYPES	C3bi-COATED MICROSPHERES	CONTROL MICROSPHERES
CHO-CR3S	76%	18%
CHO-MAC-1	53%	12%
CHO-WT	12%	15%

The functional activity of CR3 expressed in transfected CHO cells was assessed by incubating CD11b/CD18 expressing cells with microspheres coated with PGL-1, which when incubated with fresh serum activates the classical complement pathway, leading to the deposition of C3bi on the surface of the microsphere. Microspheres incubated in serum alone were used as a control.

5.4 DISCUSSION

The subcloning of the CD11b and CD18 cDNAs and their transfection into CHO cells presented a number of specific problems and difficulties. During the subcloning procedure, the use of intermediate cloning steps, as with the general cloning vector pBS, allowed us to plan the most convenient cloning strategy for the vectors of our choice, and also allowed us to sequence the cloned product and ensure that the cDNA was in the correct frame for expression. This also proved useful for mapping, as not much was known about the internal restriction sites in the two cDNAs, and we therefore had the opportunity to test various restriction enzyme digest combinations, thereby providing a more detailed restriction digest map of the two subunits for further cloning steps.

The results obtained by FACS analysis of the pΩCD11b and pΩCD18 clones were confusing and disappointing. They seem to suggest that the CHO cells had only been transiently transfected, resulting in the initial expression of the receptor, but upon subsequent selection expression was lost. This may have been due to reorganisation of the chromosomes resulting in cells containing the SV2Neo gene only, which confers survival in G418-containing media. Although our knowledge is limited about the mechanism(s) of insertion of transfected DNA into the cell chromosome, it can be speculated that although the cDNAs were integrated initially, they were not essential for the survival of the cells and may have placed too great a physiological stress on the cell, resulting in the splicing out of the CR3 cDNAs during cell division. Another explanation for the transient expression may be the lack of a stringent cloning technique, which resulted in a pooled population of selected cells at the initial level of

selection. These cells may have contained predominantly the SV2Neo minigene, because of overgrowth of these cells with respect to the CD11b- and CD18-transfected cells, which may require a longer culture period for adequate gene expression and cell division.

This would explain the initial results, but not the two subpopulations of cells seen for each clone after immunopanning. We have no clear understanding of this, but it has been suggested that cells struggling to survive in adverse conditions may become large and flat in appearance, thus giving rise to the larger cell population (Bebbington et al., 1987)..

During the lifting of the cells, an EDTA solution was used to chelate the metals essential for CAM function. This may also have affected the MAb-receptor interaction, leading to poor FACS results, as a functional heterodimer is required for correct epitope exposure. All of these interpretations are speculations, however, as we do not have sufficient data to reach a firm conclusion. Therefore, since the level of expression of CD11b/CD18 with the pΩ vector was insufficient for our requirements we turned to the pEE system for subsequent transfections. This was prompted in part by the report on high-level stable expression of CR3 in CHO cells using a methotrexate-amplifiable system (Diamond et al., 1993). Indeed, we have acquired these cells, CHO-MAC-1 cells, as a positive control for our experiments. Nevertheless, because of our interest in generating CHO cell lines expressing mutant CR3 receptors - for example, secreted CR3 with truncated transmembrane domains, CR3 with the cytoplasmic domains deleted, or single-subunit expression - we have persisted with our efforts to generate stable lines of CHO cells expressing CR3.

Using the pEECD11b and pEECD18 expression vectors we have demonstrated that functionally active CR3 is expressed on the cell surface of non-phagocytic CHO cells transfected with these constructs. As judged by indirect and direct immunofluorescence and an immunobead assay, the levels of surface expression of both subunits, CD11b and CD18, are comparable to those expressed by the CHO-MAC-1 cells (from Springer's laboratory). (Figure 5.6). Moreover, we have established that the pEECD11b/pEECD18-transfected cells (designated CHO-CR3 cells to distinguish them from the Springer CHO-MAC-1 cells) express functionally active CR3 at the cell surface, as determined by binding of C3bi-coated microspheres (Table 5.6). Consequently, both the CHO-MAC-1 cells and the CHO-CR3 cells generated here were used in the *in vitro* infection studies described in Chapter Six.

In addition to transiently transfected CHO-CR3 cells, attempts were made to establish true stable CHO-CR3 cell lines. These were generated in glutamine-deficient medium containing 25μM methionine sulfoximine (MSX). Following this, a small number of MSX-resistant colonies (approximately 8) survived, which were isolated, grown up, and two chosen for further selection. These were exposed to progressively higher doses of MSX (up to 200μM), over a period of 4-8 weeks. One of the clones resistant to 200μM MSX was chosen for further work, designated CHO-CR3S cells, and these cells were analysed for CD11b/CD18 expression and function as described.

However, at some time during the 4-month period after transfection, the CHO-CR3S cells lost the expression of CD11b. This was first noticed by RT-PCR with primers specific for the CD11b I domain, with which no specific PCR product was detected in the CHO-CR3S cells, whereas this message was clearly detected in the CHO-MAC-1 cells. Moreover, no PCR product could be amplified with the same primers from

genomic DNA from the CHO-CR3S cells, unlike the CHO-MAC-1 cells, which were positive. These results were confirmed by immunofluorescence, which failed to detect the CD11b subunit on the cell surface. However, use of CD18-specific primers revealed, by RT-PCR, that expression of CD18 was retained by the CHO-CR3S, and this subunit could be detected on the cell surface by immunofluorescence. (This work was performed in the laboratory by N. Godiner and is reported here as a personal communication.) Retention of the CD18 gene explains how these cells could remain resistant to high levels of MSX (which requires high expression of glutamine synthetase, as provided on the pEE vector).

These results indicate that, although the CHO-CR3S cells are stable transfectants, they express only the CD18 subunit, and that at some point the CD11b was lost. It is curious that CD18 can be expressed on the cell surface in the absence of CD11b, which is contrary to what was reported in human kidney 293 cells, in which only the CD11b subunit was able to reach the cell surface in the absence of its partner (Berman et al., 1993). We intend to determine by immunoprecipitation whether the CD18 is present on the surface as an isolated subunit, or more likely, whether it has dimerised with endogenous CHO cell α subunits such as α_5. (This work falls outside the scope of this thesis and will be reported elsewhere.) In the infection studies presented in Chapter six, we have used the CHO-MAC-1 cells as well as the CHO-CR3T and the CHO-CR3S cells; most of these studies were performed within 4-8 weeks of generating the CHO-CR3S cells and we know that during this time CD11b/CD18 expression was maintained.

To overcome the problems with the CHO-CR3S cells, the following modifications to the transfection protocol can be suggested. First, the pEECD11b and pEECD18

vectors should be cotransfected together with pSV2Neo, which confers neomycin resistance. This is based on unpublished observations (T. Dower and E. Sturrock, personal communications) indicating that initial selection with neomycin analogue G418 followed later by selection and amplification with MSX greatly improves the yields of transfectants. Second, mindful of the apparent instability of the CD11b gene, we should carefully monitor and select for CD11b-expressing transfectants. This can be done by enriching the fraction of CD11b-expressing cells by immunopanning with the MiniMacs magnetic bead system, using the anti-CD11b MAb OKM-1. Enriched fractions should be checked by immunofluorescence and flow cytometry for both CD11b and CD18 expression. Promising lines should then be cloned by limiting dilution in an attempt to select out a stable clone expressing high levels of the CD11b/CD18 heterodimer.

CHAPTER SIX

EUKARYOTIC CELL INFECTIONS

6.1 INTRODUCTION:

Invasion of host cells may lead to the protection or destruction of the pathogen, depending on the route of entry and type of cell invaded. There are two broad types of invasive bacteria, firstly those that are obligate intracellular pathogens, such as *M. leprae*, which are dependent on the intracellular environment for survival, and secondly the facultative intracellular pathogens, such as *M.tb* and *Yersinia spp.*, which prefer the intracellular environment, but can survive on non-living extracellular material. Bacteria must orchestrate their pathway of entry carefully, to minimise the host's defence mechanisms and to provide a niche for optimal survival and growth. Many intracellular pathogens target the monocyte-macrophage cells as their initial host cells, to aid in their dissemination via the lympho-hematogenous route. The initial evasion of host defences enables the bacteria to establish adequate infections or colonisation before the activation of the host's specific cell-mediated immune response, after which bacteria may be killed or restrained in the immune-competent host.

To enter cells bacteria need to adhere to the cell membrane, and then gain access to the cell's interior, either within membrane-bound vacuoles or free within the cytoplasm. The extent of their success is determined by the nature of the receptor that is targeted by bacteria, the strength with which the bacteria can bind to the receptor, the number

of receptors the bacteria can bind to and whether or not the bacteria can orchestrate the activation of the receptor, to induce internalisation. When examining integrin-mediated entry of bacteria into host cells, the α subunit of integrin heterodimers usually dictates the ligand specificity, whereas manipulation of the ß subunit enables direct interaction with the cell cytoskeleton and intracellular signalling pathways (Isberg et al., 1994). Often the engagement of one integrin may allow upregulation of a second integrin which in turn mediates internalisation, as seen in fibronectin (Fn) binding and the subsequent activation of CR3 (Hazenbos et al., 1993).

The association between the bacterium and the host cell may be advantageous for the bacterium, but also induces a number of functional changes in both the eukaryotic cell and the bacterium. The ability to adhere to the cell membrane enables the bacterium to avoid physical removal associated with coughing and sneezing. Bacteria have established a number of infection mechanisms that are adhesion molecule-dependent, of which the three best described strategies, are lectin binding, masking, and mimicry (Tuomanen, 1993). Lectin binding involves the adherence of bacteria to a carbohydrate moiety of a cell surface glycoprotein, as seen with pathogenic strains of *E. coli.* Masking entails the adsorption of serum proteins, such as complement component C3bi, onto the surface of the bacteria, which in turn forms a bridge to interact with the I domain of the integrin CR3, as demonstrated by *Legionella spp.* and *M. leprae*. Mimicry involves the existence of a bacterial cell surface moiety that mimics a natural integrin ligand, and therefore engages a host cell integrin directly. *B. pertussis* uses this strategy to mimic factor X's interaction with integrin CR3, by producing filamentous haemagglutinin *(FHA)* that shares sequence homology with this natural ligand (Zierler and Galan, 1995).

One of the host's most potent defence mechanisms is the ability of phagocytic cells to recognise and phagocytose foreign particles. The expression of the Fc receptor (FcR) complex aids in the recognition of antibody-coated particles, as well as providing the required link between the phagocytic effector cell and the B lymphocyte's ability to secrete Ig for opsonisation of particles. The FcRI and FcRII are structurally integrated with the cell's cytoskeleton and are therefore involved in phagocytosis. The FcRIII in contrast, is devoid of a transmembrane and cytoplasmic domain and linked to the membrane only by inositol phosphatidyl groups, suggesting that it cannot be involved in transmembrane signalling and cytoskeletal manipulation events. Recent studies have demonstrated that co-operation between receptors is an increasingly recognised phenomenon, as an activating mechanism, and to aid recognition and phagocytosis. Such complementary functions are demonstrated by the binding and internalisation of IgG-coated erythrocytes by non-phagocytic cells expressing FcRIII and CR3. Cells expressing CR3 only are unable to bind or phagocytose these particles, whereas cells expressing FcRIII only bind IgG-coated erythrocytes but cannot internalise them (Mosser, 1994; Krause et al., 1994).

Manipulation of host defences is particularly well illustrated by respiratory pathogens, which exploit protective barriers to their own advantage. In many instances these pathogens target CR3 on macrophages, (the highest expressors of CR3), but often these cells must first be recruited to the site of infection. A complex array of cytokines and chemotactic signals is dispatched by both the inflamed endothelium and the bacteria, which induce the circulating monocytes to migrate to the site of pathology. After first upregulating P-selectin and later E-selectin on the damaged endothelium of the blood vessels and lung tissue, leukocyte integrin ß subunits are activated, and

tethering, diapedesis and migration are enhanced (Tuomanen, 1993; Cooper et al., 1994). An additional component of the activation of integrins by chemotaxis are conformational changes with resultant structural exposure of new binding epitopes that may be advantageous to the bacteria at the site of infection (Cooper et al., 1994).

6.1.1 Mechanisms of Invasion:

Successful invasion strategies are best illustrated by a description of the interactions of well-studied intracellular pathogens with their respective host cells.

6.1.1.1 Opsonic Binding to Complement Receptors:

The first set of organisms to be discussed are those that utilise complement component C3bi as an opsonin that is fixed to their cell surface, by either the classical pathway or the alternative complement cascade (masking). *Leishmania spp.*, *Legionella spp.*, *Rhodoccocus equi* and *Mycobacterium leprae* are well known for their ability to enter phagocytes via the CR3-C3bi route. *R. equi*, a Gram-positive respiratory pathogen that is fast becoming a major cause of granulomatous, cavitating pneumonia in AIDS patients, binds to and invades alveolar macrophages (ALMø) in a strictly serum-dependent manner. These bacteria are able to fix complement on their surfaces by means of activation of the alternative complement pathway, and then induce rapid phagocytosis by engaging both the CR1 and CR3 (Hondalus et al., 1993). *Legionella pneumophilia*, an obligate intracellular pathogen, uses a similar strategy, initiated by its major outer membrane proteins (MOPS), to activate the alternate complement

pathway and enhance CR1- and CR3-C3bi interactions. It has recently been suggested that these bacteria are also able to interact with the CR3 in a complement-independent manner, by binding to the lectin binding site on CR3 (Bellinger-Kawahara and Horwitz, 1990; Yamamoto et al., 1994).

Leishmania major also activates the alternate complement pathway, in this case by the presence of a lipophosphoglycan (LPG) outer layer, associated with the metacyclic growth stage, and the *gp63*, a metalloproteinase, of the promastigote, which both allow interaction with CR3 and CR1. Unlike *L. major, L. mexicana* does not appear to require complement to interact with CR3, indicating different evolutionary strategies used by closely related species of the same pathogen (Titus et al., 1994).

Similarly, *Mycobacterium leprae* contains a cell wall component, phenolic glycolipid-1 (PGL-1), that actively binds complement component C3, and activates its breakdown via the alternative complement pathway, to display C3bi on its surface. This, as with *R. equi*, *Leishmania spp.* and *Legionella pneumophila,* enables direct interaction of the fixed C3bi with the I domain of the α subunit of CR3. In common with many of the pathogens that use this invasion route, a small percentage (10%) of *M. leprae* bind to macrophages in the absence of complement, suggesting that an alternative route of entry is also possible. To favour *M. leprae's* interaction with CR3, the normal down regulation of CR3 by IFN-gamma is inhibited by *M. leprae*, so as to enhance the interaction with the host cell during inflammation, suggesting a further manipulation by invading pathogens of inherent host mechanisms (Schlesinger et al., 1991b).

An accessory mechanism to C3bi binding is the deposition of fibronectin (Fn) on the surfaces of bacteria, by means of Fn-binding proteins. This in turn allows binding to

the α5ß1 (VLA-5) integrin and consequent activation of CR3, via signal transduction pathways, enabling enhanced interaction between CR3 and the pathogen. This is an interesting demonstration of the communication that exists between cell surface receptors (Isberg, 1991; Hazenbos et al., 1993).

6.1.1.2 Nonopsonic Binding to Complement Receptor Type 3:

Bordetella pertussis and *Histoplasma capsulatum* have also evolved mechanisms for binding to CR3 in a complement-independent manner. However, the epitopes used by these pathogens seem to be distinct from the C3bi-binding site on the I domain of the α subunit, but these nevertheless stimulate the internalisation mechanisms *Bordetella pertussis* is a classic host-specific and tissue-specific pathogen that has evolved to maximise its ability to adhere to lung cilia and penetrate specific host macrophages to establish a non-invasive lung infection. To mediate this highly specific interaction, *B. pertussis* produces two secreted proteins, *FHA* and pertussis toxin which interact in distinct ways to allow progression of disease. Absence of these two proteins inhibits the bacterium from binding to the host cells and enables the host to clear the bacteria from the bronchial passages by mechanical means, such as coughing.

Pertussis toxin is a 105 kDa ADP-ribosylating toxin that consists of a number of subunits, which together disable the host cell by increasing intracellular cAMP. In addition, a 921-Da tracheal cytotoxin released from the peptidoglycan of the bacteria causes destruction of the cilia, which frequently accompanies the disease process. Toxicity is not only of bacterial origin, but is also related to the ability of the bacteria to induce IL-1 and nitric oxide production by the host epithelial cells. Subunits S2 and

S3 of the pertussis toxin, which interact with host cell glycoproteins and glycolipids, target ciliated epithelial cells and human macrophages, respectively. These subunits have a high sequence similarity to mammalian C-type lectins, which may explain their specificity for these cell types. The pertussis toxin also acts synergistically with *FHA*, by upregulating the expression of CR3 on the macrophages by means of the selectin-like component of pertussis toxin which is similar to the effect of P-selectin on inflamed endothelium thereby enhancing the number of receptors available for interaction with *FHA*.

FHA is a 220-kDa secreted protein that consists of several epitopes to enhance bacterial-cell interactions. These include an N-terminal heparin binding domain that interacts with sulphated polysaccharides and heamagglutinin, an N-terminal lectin domain that binds to sialic acids and heamagglutinin, a second lectin domain that aids adherence to cilia, and an RGD domain that contains two regions that mimic binding sites for factor X, a known ligand for CR3. These *FHA* epitopes allow the bacteria to invade host macrophages without evoking a respiratory burst (by binding to CR3)and provide them with an adequate intracellular niche to promote bacterial growth, without causing tissue destruction. If the bacteria are unable to invade the host cells they remain adherent to the cilia carbohydrate moieties and continue to cause extracellular damage (Tuomanen, 1993; Cundell and Tuomanen, 1995; Cooper et al., 1994; Relman et al., 1990; Newman et al., 1990). Recent data have suggested that *B. pertussis* also binds additional integrins on the host cell, such as $\alpha 5\beta 1$, by means of Fn, thereby utilising the cell's intracellular signalling pathway to upregulate the preferred receptor CR3, again manipulating the cell's normal functions for optimum targeting (Hazenbos et al., 1993).

Histoplasma capsulatum (Hc), a well known respiratory pathogen, also enters host ALMø in a complement-independent manner, so as to establish an intracellular infection before the host cellular immune response is initiated. Hc, long thought to bind to similar receptor epitopes as zymosan, has in fact been shown to adhere to the ß2 subunit of leukocyte integrins in a temperature- and cation-dependent manner, which is distinct from the zymosan-dependent binding site of most other yeast species (Bullock et al., 1987). Extensive experiments indicate that unlike C3bi-opsonised bacteria that bind to the I domain and induce internalisation via polymerisation of the microtubules, the Hc cell surface moiety uses different cell signal pathways to interact with the actin microfilaments of the host cell cytoskeleton, which suggests that there are two independent methods of cytoskeletal manipulation by engagement of different CR3 epitopes. The method of internalisation utilised by the *Hc* may result in the limited intracellular survival of the organism, as engagement of the ß2 subunit does not protect the pathogen from a respiratory burst in immune-activated macrophages (Ross et al., 1985; Newman et al., 1990). This has been confirmed by incubation of Hc with ß2 subunit-deficient LAD leukocytes, which are unable to internalise the organism (Ross et al., 1985; Graham and Brown, 1991).

6.1.1.3 Nonopsonic Invasion of Nonphagocytic Cells:

In contrast to opsonin-dependant binding to integrin receptors, certain of the *Yersinia spp* have evolved to express invasin, a 103 kDa cell-surface protein that binds directly to members of the ß1 integrin family expressed on epithelial cells. To induce phagocytosis in non-phagocytic cells, bacteria must compete with natural ligands for

adherence, and *Yersinia*'s invasin interacts with a higher binding affinity for the ß1 integrin than fibronectin, the natural ligand, thereby favouring adhesion and subsequent internalisation. This binding allows effective manipulation of the host cytoskeleton, by inducing alterations in signal transduction pathways to allow internalisation.

Many non-phagocytic cell surfaces have defence mechanisms to prevent bacterial colonisation and penetration, such as ciliation, peristalsis and continual flushing, which mechanically decrease the physical interaction between pathogens and host cells. To overcome these non-specific defences and to ensure rapid colonisation of the host epithelium, *Yersinia* expresses high levels of invasin on initial infection to allow fast and effective adhesion and penetration. Subsequently, a second protein, produced by the *ail* gene, is expressed, induced by the increased temperature within the host, suggesting a specific involvement in cellular interactions. Moreover to prevent phagocytosis of the bacteria by circulating macrophages, *Yersinia* produce a third class of protein called *Yops*, which prevent internalisation by entering the host cell and paralysing the cytoskeleton (Isberg, 1991; Young et al., 1992; Isberg and Van Nhieu, 1994; Zierler and Galan, 1995; Bliska et al., 1993).

Salmonella typhimurium, another intestinal pathogen that invades non-phagocytic cells to establish an infection, exploits host signal transduction pathways to induce uptake. These bacteria adhere to intestinal microvilli and induce membrane ruffling at the point of adhesion, followed by internalisation. This appears to involve a complex series of events, which include depolarisation and disruption of tight junctions, intracellular calcium fluxes, activation of inositol triphosphate secretion, enhanced epidermal growth factor receptor expression, mitogen-activated protein kinase and leukotriene D_4 activation, and induction of cytoskeletal rearrangement. Once the bacterium has

invaded the cell, the cytoskeletal disturbances cease and the cell returns to normal functions (Zeirler and Galan, 1995; Finlay and Seiber, 1995; Galan, 1994).

Although the mechanisms are different, the common aim of all these intracellular pathogens is to invade the host cell successfully and to optimise conditions for replication. The manipulation and subversion of normal host cell functions by the pathogen are striking and appear to have evolved at a faster rate than the host defence mechanisms. For respiratory pathogens, the leukocyte adhesion protein CR3 seems to be the most manipulated, providing the safest route of entry and the establishment of disease.

Many studies have been designed to identify pathways of entry, but a key variable that is often overlooked, and which seems to be important in determining the true relevance of the host-pathogen interaction, is the cell type studied. As revealed by more recent studies, neutrophils, monocytes, and macrophages have different functions and abilities to recognise and respond to ligands. The same ligand may not adhere at all to neutrophils, weakly to monocytes, but strongly to macrophages, even though it targets the same receptor. This may be a function of receptor expression or conformation, i.e. epitope exposure, as dictated by host cell signalling pathways to control ligand specificity. This would be a plausible regulatory mechanism, as these integrins are not designed primarily for pathogen recognition, but mainly for host cell-cell and cell-substratum interactions. It would be disastrous if various receptors were upregulated on different cell types all the time and with the same ligand specificity. It is thus remarkable how pathogens have evolved to use these differing specificity's to their own advantage (Hondalus et al., 1993; Zhou et al., 1994; Hazenbos et al., 1993; Bullock et al., 1987; Krauss et al., 1994; Newman et al., 1990).

Numerous studies have investigated the interaction of live, virulent *M.tb.* with macrophages, but the importance and characteristics of binding to CR3 are still not clearly delineated. It is well known that *M.tb.* uses CR3 to invade host cells, but what the relative importance is of this receptor versus other macrophage receptors and whether the binding is predominantly opsonic or nonopsonic remains unclear. It is difficult to study these questions in macrophages, because these cells present a complex array of cell-surface receptors and are also able to synthesise endogenously various opsonins, including complement components, as described in Chapter 4. We have therefore decided to use CR3-expressing CHO cells as macrophage surrogates in order to answer two questions a) can *M.tb.* use CR3 alone as a receptor for adherence and invasion, and b) is complement essential for this interaction ?

We hope that these experiments can provide some new insights into the mechanisms that *M. tb.* uses to establish infection and disease.

6.2 MATERIALS AND METHODS:

6.2.1 Bacteria:

Mycobacterium tuberculosis (M.tb.) strain H37Rv (ATCC 27294) was grown as in Kirchner's broth and stock suspensions frozen at -20°C in 0.5 ml aliquots, described in Chapter 3.

6.2.1.1 Quantitation of bacteria:

Bacteria were quantitated as described previously and resuspended to provide an inoculum of approximately 12 x 10^8 organisms/ml.

Bacteria were diluted and quantitated via optical density at 550 nm with reference to a standard curve calibrated by enumeration of serially diluted bacteria stained by ZN.

Prior to infection, the bacteria were defrosted at room temperature vortexed, and then syringed to resuspend the bacteria. These bacterial suspensions contained single bacteria and small clumps of two to five organisms, as seen on ZN staining.

6.2.2 Mammalian Cells:

6.2.2.1 Monocyte derived Macrophages:

Human monocyte derived macrophages (MDMs), were obtained, from human peripheral blood mononuclear cells (PBMC) as described in Chapter 3, and grown in RPMI-1640 with 5% autologus, fresh serum and 1:10 "cytokine-containing" supernatant. Cells were cultured at 37°C, 5% CO_2 for a minimum of 5 days, before being classified as macrophages.

6.2.2.2 Transfected CHO cell lines:

CHO-WT, CHO-CR3T and -CR3S, and CHO-MAC-1 were cultured as described in Chapter 5. Cells were cultured in tissue culture flasks in complete media as

appropriate for the specific cell line (Addendum A.2.), grown to 60% confluence, and then seeded for infection studies.

6.2.2.3 Seeding of cells:

All cells were washed with PBS after removal of culture media, treated with ice cold 5mM EDTA/HBSS, and incubated at 37°C, 5% CO_2 for 5 minutes, to enable lifting of the cells from the culture dish without proteolysis of the adhesion receptors. The lifted cells were washed off the tissue culture flask with PBS, resuspended in 10 ml PBS, and transferred to 10-ml sterile plastic centrifuge tubes. The cells were pelleted at 2000 rpm for 3 minutes and washed in PBS to remove all traces of EDTA. The cell pellet was resuspended in 1 ml of PBS and prepared for counting as described in Addendum A.1.14. Cells were quantitated by a Coulter counter or heamocytometer, and resuspended at approximately 1 x 10^6 cells/ml.

Cells were seeded at 1 x 10^5 cells/coverslip onto 12-mm glass coverslips in 24 well tissue culture plates, refed with 1 ml of complete media, and incubated at 37°C, 5% CO_2 to allow adherence. Cells were left for 4 hours, to adhere. Each cell line was seeded in duplicate or triplicate per parameter per experiment.

6.2.2.4 Media Conditions:

To establish the role of complement in promoting adherence and internalisation, the cells were incubated in 50% DMEM/F12 alone or supplemented with 10% heat-inactivated (HI) FCS, 10% fresh FCS, or 10% fresh human serum. No antibiotics or selection agents were present in these media. Cells were washed in PBS 1 hour prior

to infection, to remove any debris, and then maintained in the appropriate media at 37°C, 5% CO_2.

6.2.2.5 Monoclonal Antibodies (MAbs):

MAbs added in an attempt to block binding of bacteria to CR3, were used either as hybridoma supernatants or purified from ascites fluid. Anti-CD11b antibodies included: LM2/1.6.11, a mouse IgG1 anti-human MAb from ATCC, was used as the hybridoma supernatant; 2LPM19c, a mouse IgG1 anti-human MAb from Dako, and YFC118.3, a rat IgG2b anti-human MAb from Serotec, were purified from ascites fluid. The anti-CD18 MAbs were all mouse IgG1 anti-human MAbs: TS1/18.1.2.11 from ATCC was used as the hybridoma supernatant, whereas MHM23 and Clone 44, from Dako and Serotec, respectively, were purified from ascites fluid (McMichael, 1987).

6.2.3 Infections:

The cells, incubated in their appropriate media, were infected with between 5 and 50 µl of bacterial Kirchner's suspension, depending upon the bacterial concentration, to achieve infection ratios of 50:1 and 500:1, bacteria to cells, for the MDMs and CHO cell, respectively. The tissue culture plates were swirled briefly to distribute the bacteria, and incubated at 37°C, 5% CO_2 for 24 hours or less depending upon the

experiment. All work with live, virulent strains of *M. tb.* was performed in a modified P2 facility as described in Chapter 3.

6.2.3.1 Antibody Inhibition:

Wells containing cells seeded onto glass coverslips were washed after 4 hours incubation to remove the serum-containing media and 25-30 µg/ml of each MAb was added per well and incubated for 30 minutes at 37°C, 5% CO_2, after which the appropriate culture media were added and the cells infected at a 500:1 bacterial/cell ratio with H37Rv, as described above. To pre-coat the coverslips, 25-30 µg/ml of the MAb was added to each coverslip and incubated for 3 hours at 4°C. The coverslips were washed with PBS containing 1% BSA and again with PBS only, before allowing the cells to adhere (Rozdzinski and Tuomanen, 1994; Newman et al., 1990). When hybridoma supernatants were used, no attempt was made to determine the concentration of the MAb. Supernatants were used at 250 µl per ml of culture media in all experiments. Cells were incubated with the bacteria for 24 hours, stained and fixed, and the percentage of cells associated with bacteria determined as described below.

6.2.4 Fixing and Staining of Infected Cells:

The cell cultures were removed from the incubators and washed at least three time with room temperature PBS, to remove any non-adherent bacteria and dead cells. The cells were fixed in 2.5% glutaraldehyde (GA), and kept at 4°C for 60 minutes before staining for microscopy.

Cells were washed again with room temperature PBS to remove the GA and flooded with 1.4 mg/10 ml acridine orange for 1 minute at room temperature, and then washed with PBS to remove excess acridine orange. To quench the extracellular fluorescent bacteria, 5 mg/10 ml of crystal violet was added to the coverslip for 30 seconds at room temperature, and then removed with PBS. Coverslips were inverted onto glass microscope slides and adhered with clear nail varnish. The slides were kept at 4°C in the dark for less than one hour before examination under fluorescence microscopy.

6.2.4.1 Quantitation of Associated Bacteria:

One hundred to two hundred cells were examined at random per coverslip to quantitate the association of fluorescent bacteria with the cells. The results were expressed as the percentage of cells associated with bacteria. Slides were analysed with a Zeiss Axiovert fluorescence microscope fitted with a 100x acromat planar lens.

6.2.5 Electron Microscopy:

To determine qualitatively whether the bacteria were intracellular, rather than extracellular, both CHO-WT and transfected CHO cell lines were examined by transmission electron microscopy (TEM). The cells were infected as described, incubated for 24 hours, then lifted in 5 mM EDTA/HBSS and fixed in a cacodylate buffer containing 0.1 M sucrose, 5 mM Ca^{2+} , 5 mM Mg^{2+} and 2.5% GA, at 4°C overnight. The cells were then washed twice with the cacodylate buffer and the cell

Table 6.1: *Mycobacterium tuberculosis* Binds to CR3-expressing CHO cells in the Presence and Absence of Serum

SERUM	% CELLS THAT BOUND *M. tb.*		
	CHO-WT	**CHO-CR3S**	**CHO-MAC-1**
Fetal Bovine Serum			
Fresh	14 ± 3	77 ± 5	80 ± 9
None	17 ± 6	79 ± 6	79 ± 4
Human Serum			
Fresh	15 ± 2	81 ± 6	57 ± 6
Heat-inactivated	16 ± 3	79 ± 3	58 ± 5
None	19 + 5	82 ± 8	69 ± 7

CHO-WT, CHO-CR3S, and CHO-MAC-1 cells were incubated with *M. tb.* (300:1), washed, stained, and the number of cells that associated with one or more bacteria counted and expressed as a percentage. Values are means ± SD derived from 5 (bovine serum) and 5 (human serum) separate experiments, where n = 3-9 per cell line per experimment.

pellet was stained with 1 % osmium tetroxide in a sucrose-free cacodylate buffer for 1 hour before embedding in resin. Sections were examined on a Hitachi H600 TEM.

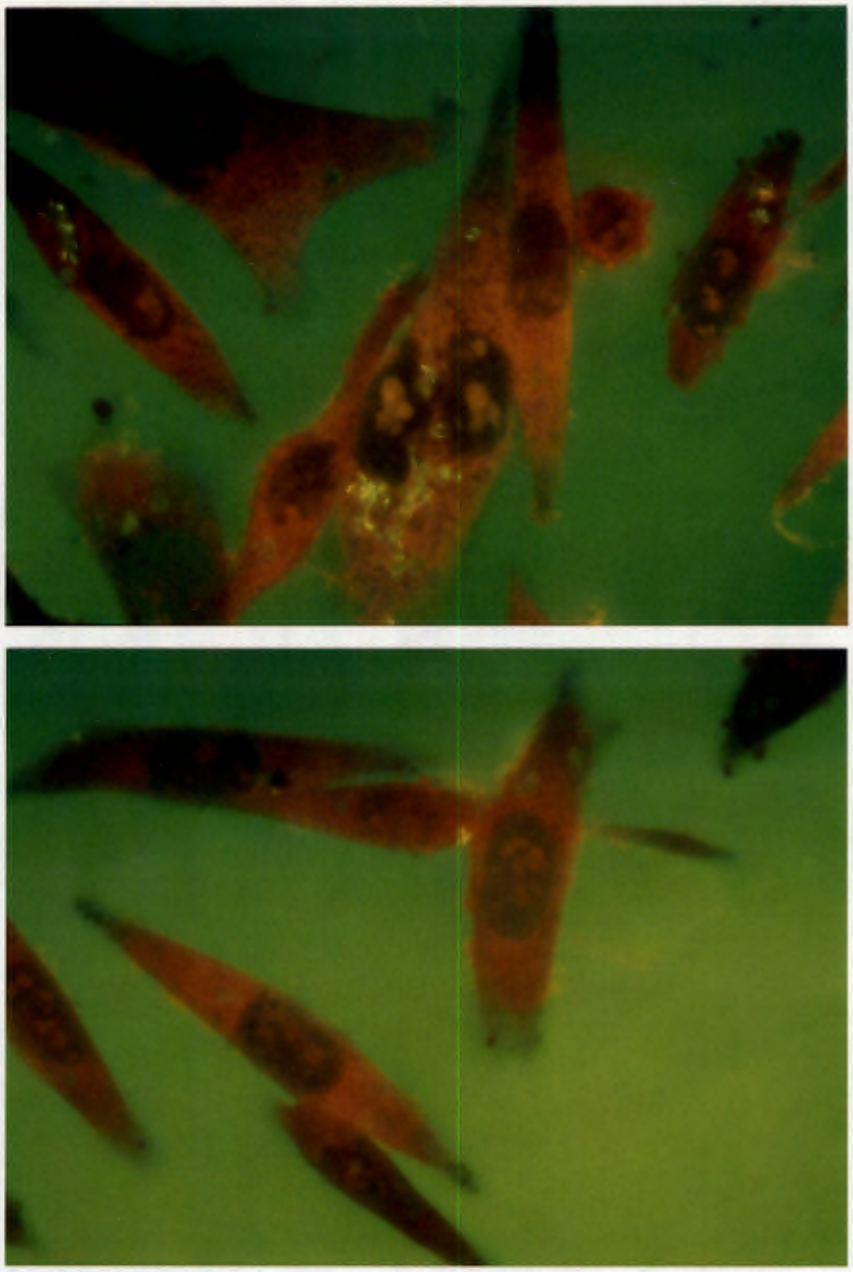
6.3 RESULTS:

6.3.1 Binding of *M.tb.* to CR3-Expressing CHO Cells:

Tranfected and untransfected CHO cells were infected with *M.tb* as described. Each coverslip was examined under fluorescence microscopy to determine the percentage of cells associated with the bacteria. No attempt was made to distinguish between intracellular and extracellular bacteria. The infections were performed in various media conditions as discussed, i.e.. supplemented with fresh serum, heat-inactivated serum, or no serum.

The MDM cells were infected in much the same way, but at a far lower infection rate, because of the greater phagocytic ability of these cells. At ratios higher than 50:1 bacteria to MDM, bacteria filled the cell to capacity, which often resulted in cell lysis. The infections were performed in media conditions comparable to those used with the transfected cells. The infection ratios for MDMs are comparable to reports in the current literature. In contrast to infection ratios of 50:1 for the MDMs, ratios up to 500:1 were used with the CHO cells, which we found was necessary to achieve useful results with this non-phagocytic, fibroblast cell line.

Figure 6.1: Infection of CHO-CR3 cells and CHO-WT with *M.tb.*



(A) CHO-CR3 infected cells indicate large numbers of acridine orange stained *M. tb.* associated with the cells, in comparison to the few bacteria seen in (B) CHO-WT infected cells, suggesting the dependence of CR3 for bacterial-cell association.

The two most striking results of the binding of *M. tb.* to CHO cells were, (a) binding of *M. tb.* to CR3-expressing CHO cells was consistently 5-fold greater than to wild-type CHO cells; and (b) the binding of *M. tb.* to CR3-expressing CHO cells was essentially identical in the presence of fresh serum, heat-inactivated serum, or no serum (Table 6.1and Figure 6.1). Overall, in 7 separate experiments, the percentage of CR3-expressing CHO cells that bound *M. tb.* was 67 ± 13% in the presence or absence of serum, versus 14 ± 3% of wild-type CHO cells. Specifically, in the presence of 10% fresh FCS, bacteria associated with 77 ± 5% (1.25 ± 0.21 bacteria/cell) of CHO-CR3T, 80 ± 9% (1.95 ± 0.77 bacteria/cell) of CHO-MAC-1, and only 14 ± 3% (0.18 ± 0.05 bacteria/cells) of CHO-WT cells. Similar results were obtained in infection studies with no serum present: 79 ± 6% (1.59 ± 0.35 bacteria/cell) of CHO-CR3T, 79 ± 4% (1.87 ± 0.50 bacteria/cell) of CHO-MAC-1 cells, and 17 ± 6% (0.18 ± 0.07 bacteria/cell) of CHO-WT were associated with bacteria in these assays, suggesting that serum opsonins are not required for the interaction of *M.tb.* with CR3 expressed in CHO cells (Table 6.1).

A second set of infections performed in the presence of human serum gave similar results. In the case of fresh human serum, HI human serum and no serum, respectively, 56 ± 5% (1.04 ± 0.15 bacteria/cell), 58 ± 4% (0.95 ± 0.21 bacteria/cell), and 66 ± 4% (1.60 ± 0.32 bacteria/cell) of CHO-MAC-1 were associated with bacteria, and under the same conditions, 15 ± 2% (0.20 ± 0.05 bacteria/cell), 16 ± 3% 0.15 ± 0.08 bacteria/cell), and 17 ± 4% (0.25 ± 0.09 bacteria/cell), respectively, of CHO-WT cells were associated with bacteria (Table 6.1). No differences could be

Table 6.2: Binding of *Mycobacterium tuberculosis* to CR3-expressing CHO cells is Inhibited by Anti-CR3 Monoclonal Antibodies

MONOCLONAL ANTIBODY	TARGET ANTIGEN	% CELLS THAT BOUND *M. tb.*				% INHIBITION
		CHO-WT	CHO-CR3T	CHO-CR3S	CHO-MAC-1	
None		18 ± 5	84	75 ± 5	67	
Mouse IgG		13		69		
2LPM	CD11b	15		79		0
LM2	CD11b	14		31		59
2LPM & LM2	CD11b	16	31			63
2LMP, LM2, MHM23 & TS1	CD11b/CD18	16 ± 6	30	15	26 ± 13	68*

CHO cells were preincubated with the indicated MAbs before incubation with *M. tb.* (300:1). The number of cells to which one or more bacteria were bound is expressed as a percentage, and the data are drawn from 6 separate experiments. Values are expressed as means ± SD (4-7 individual determinations) or only as means (2 determinations). * combined averages for CHO-CR3T, -CR3S, and -MAC-1 cells.

identified between fresh and HI serum, suggesting that no additional opsonins present in either serum, were able to enhance the bacteria assocciation to the cells.

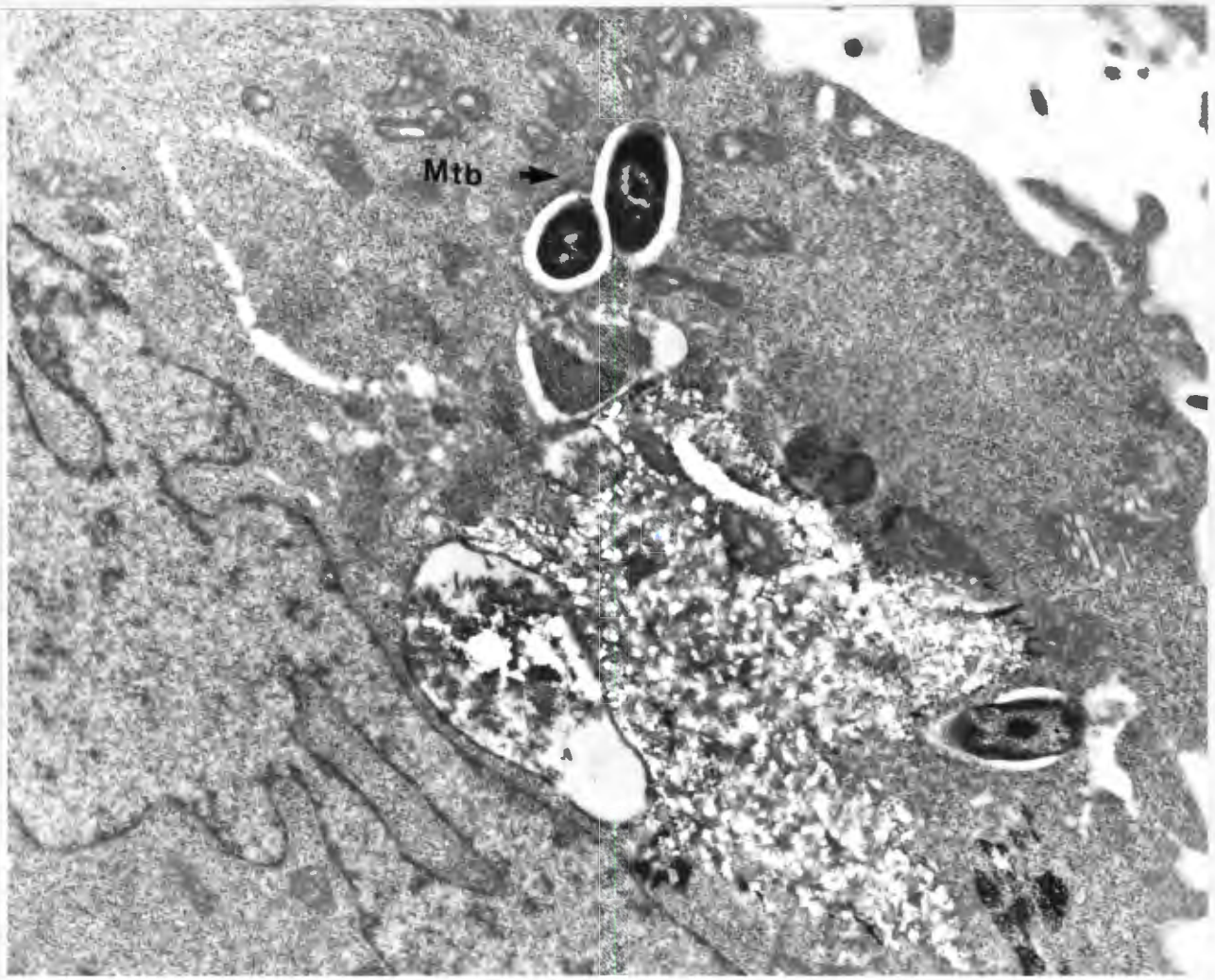
6.3.2 MAb Inhibition of Association:

The specificity of the binding of *M. tb.* to CR3 in the transfected CHO cells was confirmed by the use of MAbs to human CR3. Pre-treatment of transfected CHO cells with a combination of anti-CD11b and anti-CD18 MAbs, or adherence of the cells onto MAb-coated coverslips, reduced the binding of *M. tb.* to the CR3-expressing CHO cells by an average of 68%, but had no effect on the binding of *M. tb.* to the CHO-WT cells (Table 6.2). Pre-treatment with anti-CD18 MAb MHM23 alone reduced binding by 37%. Of the anti-CD11b MAbs, pre-treatment with MAb 2LPM19c alone resulted in no inhibition of binding, whereas MAb LM2/1.6.11 inhibited binding by 59% (Table 6.2). MAb 2LPM19c binds to an epitope that includes the C3bi-binding site (Diamond et al., 1993), suggesting that *M. tb.* does not bind to the C3bi-binding site in the absence of complement, but rather to an epitope distinct from the C3bi site. Results were similar whether the cells were preincubated with MAbs or seeded onto MAb-coated coverslips.

6.3.3 Electron Microscopy :

Infected CHO-MAC-1 cells contained numerous intracellular bacteria, either singly or in small clumps, that reside in large, membrane-bound vacuoles (Figure 6.2), an appearance that resembles that seen in infected mononuclear phagocytes. In contrast,

Figure 6.2: Electron micrograph of CHO-MAC-1 cells infected with *M. tb.*



CR3 expressing CHO cell containing single bacteria in well-defined phagosomes with seemingly tight opposing phagosome membranes, similar to those seen in MDMs infected with *M. tb.*

the CHO-WT cells contain few intracellular organisms, although the vacuole seems similar as to that seen in the CHO-MAC-1 cells. From these micrographs it is evident that a proportion of the *M. tb.* bound to CHO-MAC-1 cells is internalised and resides in a vacuolar compartment that is presumably equivalent to a phagosome.

6.4 DISCUSSION:

In recent years, the literature has been studded with descriptions of mechanisms used by pathogens to gain entry into mammalian cells. As discussed, this provides the pathogens with the opportunity to invade host cells and establish an infection. The exploitation of host cell surface integrins, seems to occur either by direct interaction with the α or ß subunit of the receptor, or by prior opsonisation with a natural ligand for the receptor. The initial specificity of binding by the pathogen will dictate the subsequent host response and cellular events, resulting either in effective disposal of the pathogen or in successful invasion of the host cell.

Predominant binding to the α subunit epitopes, seems to provide the pathogen an easy and safe passage into the cell, without the involvement of host defence mechanisms such as a toxic respiratory burst that usually accompanies internalisation of opsonised foreign particles. Bacteria that bind only to the C3bi epitope or the lectin-binding site on CR3 fail to initiate this toxic response. Presumably adequate adherence to these sites will involve signalling to the ß subunit, which is in direct contact with the host cell cytoskeleton, and allow internalisation. In contrast to this mode of entry, pathogens that interact directly with the ß subunit, such as Hc, are not as effective in evading host defence mechanisms, as a respiratory burst does accompany this interaction, but the

defence mechanisms, as a respiratory burst does accompany this interaction, but the pathogens are nevertheless able to survive in this toxic environment and establish infection (Bullock and Wright, 1987). Stimulation of the respiratory burst in this instance may not be solely the result of binding to the β subunit of CR3, but instead may reflect binding to additional cell surface receptors, such as LFA-1 and CR4, or could possibly be communication between CR3 and the FcRIII, which has been described previously to result in the respiratory burst, even though binding to either receptor alone does not elicit this response (Newman et al., 1990; Bullock and Wright, 1987; Zhou et al., 1994; Zhou et al., 1993; O'Toole et al., 1994).

The affinity with which the pathogen interacts with the host receptor and the number of receptors engaged may dictate the type of phagocytosis involved and, in fact, whether the pathogen is able to invade at all or merely remain attached to the host cell surface. As described previously, *Yersinia's* invasin protein binds with a higher affinity to β1 integrins than the natural ligands, which induces the host cell receptor to interact with the bacterial ligand, thereby encasing the bacterium in a pseudopod sleeve. This interaction was first described by Griffin and Silverstein (1974), as "zippering" phagocytosis. Although this may be so for most opsonised particles that interact with complement receptors or the FcR, other pathogens, such as *Salmonella*, have evolved to interact directly with the host cytoskeleton by influencing signal transduction pathways and triggering cytoskeletal rearrangement, by which they are engulfed. Aptly, this mode of entry is referred to as "triggering" phagocytosis (Swanson and Baer, 1995). Both these mechanisms result in internalisation of pathogens, but it has been suggested that "zipper" phagocytosis may in general be more destructive for the

pathogen, providing an excellent rationale for *Yersinia's ail* gene, which protects the bacteria after entry (Isberg and van Nhieu, 1994).

Use of various complement receptors by intracellular pathogens has been known for many years, but the molecular details of this mode of entry are only now being elucidated, as described by studies on *Histoplasma capsulatum, Legionella spp.* and *Yersinia spp.* In contrast to other CRs, CR3 has been identified as a privileged receptor, in that it allows pathogens to gain entry in to the cell without evoking the respiratory burst, thus giving the bacteria a better chance of establishing an infection (Hynes, 1992; Isberg and van Nhieu, 1994).

The study of pathogen interactions with integrin heterodimers has led to the discovery of many novel proteins and mechanisms in both the pathogen and the host, but the mode of binding of *M. tb.* to CR3 has not yet been clearly defined, other than that this may depend on complement coating of the bacteria. Binding to CR3 frequently depends on deposition of a host protein on the bacterial surface, thereby indirectly providing a ligand that binds to the receptor. Unlike the active C3bi-masking of *M. leprae, Legionella* and *Leishmania*, no surface or secreted moiety has been identified in *M. tb.* that can activate the alternate complement pathway to deposit C3bi on the bacterial surface. This suggests that *M. tb.* is opsonised non-specifically like most foreign particles, rather than an active process initiated by the pathogen. More recently, bacteria have been identified that interact directly with CR3. These bacteria express proteins, such as the *FHA* of *B. pertussis*, on their surface that bind directly to exposed receptor epitopes, leading to tight binding of the bacteria to CR3, with subsequent manipulation of the receptor. In some cases of non-opsonic binding to CR3, such as by Hc, ß-glucan and zymosan, the ligands bind to epitopes distinct from

the I domain, which seem to involve the ß subunit, as significant inhibition of binding and entry occur after treatment with ß-specific MAbs. These complement-independent ligand-receptor interactions appear to be independent of microtubule polymerisation, but instead are dependent upon actin microfilament involvement (Bullock and Wright, 1987; Zhou et al., 1994; Newman et al., 1990).

We decided to examine the documented phenomenon that *M.tb.* can enter mononuclear phagocytes in the absence of complement at up to 30% of the efficiency achieved in the presence of opsonins (Schlesinger et al., 1991a; Schelsinger et al., 1990b). Between 10 and 30% of this non-opsonic binding may be to CR3. The hypothesis that CR3 may be the receptor that is partly responsible for non-opsonic binding to *M. tb.* is plausible, as mononuclear phagocytes are high expressors of CR3, which is activated by their migration to the site of pathology, or could possibly be induced by the presence of antigens secreted by the bacilli to upregulate the expression and migration of phagocytes, in much the same manner as pertussis toxin affects these processes (Tuomanen, 1993; Cundell and Tuomanen, 1995). Moreover, a number of unrelated pathogens have been shown to bind non-opsonically to CR3, including *B. pertussis* and *H. capsulatum* (Lee and Falkow, 1990).

The experiments described in this chapter indicate that *M.tb.* can bind to CR3 in a complement-independent manner, and that this binding leads to invasion of mammalian cells. Not only was the bacterial association 5-fold greater with CHO cells expressing CR3, but in this system the ability to associate was unaltered by the absence of exogenous complement, suggesting that in these *in vitro* conditions the bacteria can interact directly with a receptor epitope.

To confirm that the binding of *M. tb.* to the transfected cells is indeed mediated by the recombinant CR3, it is important to specifically inhibit binding of *M. tb.* to CR3-expressing CHO cells. Preincubation with MAbs raised to various epitopes of the receptor provided us with useful information, not only about the receptor and its binding sites, but also about the ligand and its interaction with the receptor (Wright et al., 1983; Diamond et al., 1993). Based on the reduction in binding observed with MAbs, our initial observation is reinforced, that *M. tb.* can bind to CR3-expressing CHO cells in the absence of complement, and that this binding is to the recombinant CR3, as it is specifically inhibited by peincubation of the cells with anti-CD11b and/or anti-CD18 MAbs. Our results indicate further that the epitope is distinct from the C3bi-binding site and therefore is not in direct competition with complement-mediated binding, as similarly described in resident murine macrophages (Stokes et al., 1993). This is inferred from the use of MAb 2LPM19c, which binds to the C3bi-binding epitope and which failed to inhibit the binding of *M. tb.* to CR3-expressing CHO cells. These data suggest that the bacteria are able to bind directly to the activated CR3, by means of a surface component expressed by *M. tb.*

The significance of complement-independent binding of *M. tb.* to macrophage-expressed CR3 *in vivo* remains to be determined. Although various *in vitro* infection studies with macrophages have clearly shown a strong serum- and complement-dependent enhancement of *M. tb.* binding (Schlesinger et al., 1990b), the reported data do not allow a clear distinction to be made between the relative contributions of CR3, CR1, or CR4 to this binding. Moreover, *in vivo,* in the complement-poor alveolar space, complement-independent binding may be significant during the initial infection, and binding to CR3 may constitute the most advantageous portal of entry for *M. tb.*

into the macrophage, giving the organism an early advantage in establishing a persistent infection.

A component of nonopsonic binding may be mediated by the mannose receptor (Schlesinger, 1993), although this is limited to *M. tb.* strains with mannose-capped lipoarabinomannan moieties (Schlesinger et al., 1994), and may be restricted to resting macrophages, as activated macrophages downregulate expression of this receptor (Harris et al., 1992; Mokoena and Gordon, 1985). However, expression of the mannose receptor may be upregulated in macrophages stimulated with surfactant protein A, which results in enhanced phagocytosis of *M. tb.* (Gaynor et al., 1995). Moreover, complement-indepenndent binding of *M. tb.* to alveolar macrophages may also be mediated in part by direct opsonization with surfactant protein A (Downing et al., 1995). Nonopsonic binding to CR3 has been claimed for a number of different pathogens, including *M. tb.*, based on *in vitro* studies with macrophages in the absence of exogenous complement (Mosser et al., 1992), but these data may be spurious because the macrophage synthesises complement component C3 itself (Blackwell et al., 1985; Ezekowitz et al., 1985; Wozencraft et al., 1986). Indeed, a rigorous reanalysis of the binding of *Leishmania* spp. to CR3, previously thought to occur directly in the absence of complement, revealed that complement-independent binding of *Leishmania* to CR3 is very unlikely (Mosser et al., 1992). There are, however, at least two examples of pathogen ligands that bind directly to CR3 in the absence of complement: filamentous hemagglutinin from *Bordetella pertussis* (Relman et al., 1990) and neutrophil inhibitory factor from the hookworm *Ancylostoma caninum* (Mayer, 1961; Rieu et al., 1994). There is therefore no reason why such a ligand should not exist in *M. tb.*

One could at this stage speculate that analogous to *Yersinia* or *B. pertussis*, *M. tb.* expresses high levels of a surface protein upon entry of the host, which interacts directly with the non-C3bi epitope of CR3 on alveolar macrophages in the absence of opsonins like C3bi, and induces cytoskeletal rearrangements and subsequent internalisation, possibly by the "zipper" mechanism. Moreover, after internalisation this surface moiety may serve as a tight regulator of the phagosome, preventing lysosomal fusion or phagosomal maturation, thereby protecting the bacilli and allowing growth..

The fact that we have removed CR3 from its normal environment in the mononuclear phagocyte, where it is able to interact with other receptors, such as CR1, MR, and FcR, simplifies the analysis by removing competing receptors, but it does make the system artificial. A potential complication that is introduced in our model are uncontrolled influences of the cell type (in this case CHO cells) on the expression and phagocytic ability of CR3, as well as the epitopes exposed (i.e., the activation state), which may not be available for interaction under normal circumstances. Similarly, the intracellular signal transduction pathways present in CHO cells may affect the ligand specificity and affinity in a way different from that seen in the macrophage. A theoretical possibility, therefore, is that the non-opsonic binding observed here between *M. tb.* and CR3 is a consequence of a CHO cell-dependent functional conformation of CR3 that is not attained in macrophages. However, the use of CHO and other non-phagocytic cells (eg. COS cells) as heterologous hosts for CR3 in studies aimed at defining parameters involved in pathogen binding has been described by others, for example in experiments with *Rhodococcus equi* and *Leishmania spp.* (Hondalus et al., 1993; Titus et al., 1994). Clearly, data derived from such model

systems must be interpreted with caution, especially when extrapolating to the *in vivo* situation. Nevertheless, such studies, by isolating a phagocytic receptor of interest, provide insights into possible modes of interaction that are ambiguous when studied in the complete *in vivo* setting.

Despite these conclusions, our result that serum opsonization was completely ineffective in enhancing the binding of *M. tb.* to CHO-CR3 cells was unexpected. A careful analysis of the literature reveals that it has been clearly established that serum, and specifically C3, opsonization enhances the binding of *M. tb.* to mononuclear phagocytes, and that serum-enhanced binding is mediated by complement receptors, including CR3 (Schlesinger et al., 1990b; Schlesinger, 1993; Hirsch et al., 1994). However, as noted before, in some studies it was also noted that *M. tb.* can bind nonopsonically to monocyte/macrophage complement receptors, predominantly CR3 (Stokes et al., 1993; Schlesinger, 1993), although no attempt was made to control for macrophage-derived C3. It is evident from these results that the extent of nonopsonic binding to CR3 specifically can be difficult to dissect in studies with mononuclear phagocytes because C3 deposition leads to enhanced binding to multiple complement receptors, and addition of anti-CR3 antibodies blocks both opsonic and nonopsonic binding to CR3. Nevertheless, antibodies such as Leu-156, which is specific for the CD11b C3bi-binding site, moderately block the binding of opsonized *M. tb.* to MDMs (by 34-39%) (Schlesinger et al., 1990b), and therefore it it reasonable to conclude that a component of the binding of *M. tb.* to MDM CR3 is opsonic. We can offer two explanations for the apparent discrepancy between this conclusion and our result that binding of *M. tb.* to CHO cell-expressed CR3 is completely serum independent. First, the opsonin dependence of binding to CR3 may be strain dependent. Stokes et al.,

(1993) observed considerable variations among different *M. tb.* complex strains and other mycobacterial species in the extent of nonopsonic binding to murine macrophages. Moreover, Antal et al., (1992) noted that the C3-independent binding of group B streptococci to CR3 is likely strain dependent. The reported studies on *M. tb.* binding to monocyte/macrophages complement receptors were performed predominantly with Erdman strain (Schlesinger et al., 1990b; Schlesinger, 1993) or the H37Ra strain (Hirsch et al., 1994) grown in rich media (7H9 broth). Our studies were performed throughout with the H37Rv strain grown in a minimal medium (Kirchner's broth). We propose that in our studies, *M. tb.* strain H37Rv expresses an endogenous CR3 ligand, which binds CR3 with similar or greater affinity than C3bi, since binding is not augmented by opsonization in serum. Second, it is possible that the heterologous expression of CD11b/CD18 in CHO cells confers cell-specific activation signals on CR3 that enable the receptor to bind *M. tb.* nonopsonically to an exyenet not typical in its native environmment, the mononuclear phagocyte. This is a theoretical consideration that arises from the observeation by cell-specific signals that dictate both their affinity state as well as adhesion-modulating postreceptor events (Peter and O'Toole, 1995; O'Toole et al., 1994). The quantitative significance of this possibility can only be formally evaluated once the putative *M. tb.* CR3-binding ligand has been isolated in homogeneous form and its ability to bind macrophage-expressed CR3 has been determined. It should be noted, that the CHO-Mac-1 cells ysued here have been studied extensively with respect to the binding of four dostinct CR3 ligands, and no anomalies were detected (Diamond et al., 1993). Moreover, these and related CHO cell transfectants were used to study the nonopsonic binding of zymosan to CR3, and again no significant differences were observed in comparison to leukocyte-expressed

CR3 (Thornton et al., 1996), making this possibility unlikely. Nevertheless, in a study with murine macrophages, the nonopsonic binding of *M. tb.* was strongly dependent on macrophage phenotype, even though the binding was mediated predominantly by CR3 and levels of CR3 expression were comparable in each of the macrophage populations studied (Stokes et al., 1993). These results led the authors to conclude that the CR3 epitope binds *M. tb.* nonopsonically exists in a phenotype-dependent active or inactive state (Stokes et al., 1993). It is therefore possible, similarly, that in transfected CHO cells CR3 is expressed with the nonopsonis, *M. tb.* -binding epitope constitutively active. A detailed survey of human macrophages of various phenotypes has to be undertaken in regard to phenotype-dependence of nonopsonic binding to *M. tb.* An additional conideration relevant to the question of the functional equivalence of recombinant CR3 expressed in CHO cells compaared with macrophages CR3, arises from the recent observation that CR3 associates with glycosylphosphatidylinositol-linked proteinssuch as CD14 and CD16b in neutrophils and urokinase plasminogen activator receptor in monocytes (Petty and Todd, 1996). This association involves the CR3 β-glucan lectin site (Petty and Todd, 1996; Krauss et al., 1994), and it is therefore possible that if the nonopsonic binding of *M. tb.* to CR3 involves this or a related site, then a subset of CR3 on monocyte/macrophages is unavailable for nonopsonic binding, unlike in transfected CHO cells in which CR3 likely does not form such multi-protein complexes. Interestingly, the CR3/CD14 complex in neutrophils dissociates upon cell spreading and adherence (Petty and Todd, 1996) which, it can be speculated, may provide an explanation for the phenotype-dependence of the nonopsonic binding to *M. tb.* to murine macrophages (Stokes et al., 1993).

Unidentified at present is the presumed endogenous *M. tb.* ligand that binds directly to CR3. Although we have raised the possiblity that the binding of *M. tb.* to CR3 may involve the CR3 lectin site, this is at present speculation. Schlesinger et al. (1990b) have shown that the attachment and ingestion of Erdman strain of *M. tb.* by human monocytes was not inhibited by laminarim, a soluble β-glucan. It is possible that if other strains of *M. tb.* are examined, or if a wider range of soluble sugar antagonists of the CR3 lectin site (Thornton et al., 1996) are tested, that an inhibitory effect is observed. The sugar specificity of the CR3 lectin site is broader than originally suspected and includes mannose-containg polysaccharides (Thornton et al., 1996), which are prominent in the mycobacterialcell wall, and thuis this heparin warrants further investigation. It has been shown recently that CR3 also binds to heparin and heparin sulfate glycans. The lectin site mediating this binding is istuated in the I domain and therefore appears to be distinct from the β-glucan site (Diamond et al., 1995). It is doubtful that *M. tb.* binds to the heparin site, since this site is inhibited strongly by MAb 2LPM19c and weakly by LM2/1 and OKM1 (Diamond et al., 1995), which is the reverse of what we have found for inhibition of the binding to *M. tb.* to CHO-CR3 cells. Nevertheless, the *M. tb.* cell wall is rich in diverse saccharides, including glycolipids, peptidoglycolipids, glycoproteins, and acyl trehalose 2'-sulfates (sulfolipids) (Brennan and Nikaido, 1995), and it is conceivable that one or more of these can function as a ligand for one or the other CR3 lectin site. Notwithstanding these considerations, we can not exclude the possibility that *M. tb.* expresses a protein that functions as a ligand for CR3.

CHAPTER SEVEN

DISCUSSION AND CONCLUSION

The use of adhesion receptors is a recurring theme among intracellular pathogens, and mycobacteria are no exception. Mycobacteria are part of a phylogenetically diverse group of organisms that bind to adhesion molecules of mononuclear phagocytic cells. These adhesion proteins play an essential role in cell-cell and cell-extracellular matrix interactions, but are subverted by pathogens to allow entry into the intracellular milieu without evoking defence mechanisms, such as the toxic respiratory burst (Wright and Silverstein, 1983a), which usually accompanies bacterial entry. The specific pathogens may differ, but the process of invasion almost invariably involves manipulation of host cell signal transduction pathways and cytoskeletal rearrangements (Falkow et al., 1992; Bliska et al., 1993). The ß2 integrins CR3 and CR4 are used by *M.tb.* for adherence to host cells (Schlesinger et al., 1990a and b; Stokes et al., 1993; Hirsch et al., 1994); these integrins constitute a family of adhesion receptors intimately involved in the migration and extravasation of lymphoid and myeloid cells (Hynes, 1992; Larson and Springer, 1990). In addition to its cell adhesive functions, CR3 is the primary phagocytic receptor for C3bi-opsonised particles (Larson and Springer, 1990), as well as for the ligands ICAM-1, fibrinogen and factor X (Diamond et al., 1993). CR4, the second adhesion receptor that binds C3bi (Diamond et al., 1993), is less well understood, but may play an important role in different phenotypes of phagocytic cells, as suggested by Hirsch et al. (1994). Although CR4 may be dominant in alveolar macrophages, it is interesting that CR3 is apparently the preferred portal of entry for

many pathogens, including mycobacteria, *Leishmania* spp., *Histoplasma capsulatum*, and *Bordetella pertussis* (Falkow et al., 1992, Bullock and Wright, 1987; Newman et al., 1990). The use of CR3 may be favoured by these pathogens because it allows them to avoid the respiratory burst and provides entry into a safe niche before activation of cell-mediated immunity in the host (Relman et al., 1990; Hondalus et al., 1993). This evasion of host defence mechanisms does not seem to hold true for entry via other integrins, for example, LFA-1 and CR4, as these are accompanied by destruction of the ingested pathogen (Berton et al., 1992).

The ability of *M.tb.* to adhere to CR3 has been demonstrated in a number of previous reports (Schlesinger et al., 1990b; Schlesinger, 1993; Stokes et al., 1993; Hirsch et al., 1994), but its ability to bind to CR3 in a non-opsonic fashion still remains to be established conclusively. The complement-independent binding of *M.tb.* to CR3 would provide a mechanism for the rapid invasion of alveolar macrophages in the complement-poor environment of the lung, and is therefore potentially of real importance (Stokes et al., 1993; O'Niell et al., 1984). The mannose receptor (Schlesinger, 1993) plays a role in mediating non-opsonic binding of *M.tb.* to phagocytic cells, but this interaction seems to be dependent upon strains of *M.tb.* that express mannose-capped lipoarabinomannan (Schlesinger et al., 1994), and may only occur in resting or bystander macrophages, as the mannose receptor is down regulated in activated phagocytes (Mokoena and Gordon, 1985) and constitutively activated alveolar macrophages (Adams and Hamilton, 1992). Non-opsonic adherence to phagocytes has been claimed for many pathogens (Mosser et al., 1992), but most of these claims are based on studies done *in vitro* with macrophages, which are thought to be able to produce their own opsonic components in the absence of exogenous

complement (Wozencraft et al., 1986). Recently, *Leishmania* spp., a protozoan once thought to interact with CR3 non-opsonically, has been demonstrated to be complement-dependent in more rigorous assays (Mosser et al., 1992).

However, the non-opsonic CR3 interactions by *Bordetella pertussis* (Relman et al., 1990) and the hookworm *Ancylostoma caninum* (Moyle et al., 1994) appear to be undisputed, and therefore these examples allow the real possibility that non-opsonic binding to CR3 may be the initial route of entry for *M.tb.* into mononuclear phagocytes. To study this phenomenon rigorously, in the face of equivocal existing data, we have expressed CR3 in non-phagocytic CHO cells, so as to avoid the complicating factors encountered with endogenously synthesised complement and competing macrophage receptors for *M.tb.*

Our results have established that, first, CR3 is a receptor for *M.tb.*, and second, that the CR3-*M.tb.* interaction may occur in a complement-independent fashion. The finding that there is no real increase in adherence in the presence of serum is, on the face of it, unexpected, as enhancements of between 3-4 fold (Schlesinger et al., 1990b; Schlesinger, 1993) and greater (Hirsch et al., 1994) have been recorded in the presence of 2 to 70% serum for the binding of *M.tb.* to human mononuclear phagocytes. This increase in opsonic adherence could be due to the presence of CR1, CR4 and perhaps other receptors found on human mononuclear cells. It is possible that in our CHO cell system, the CR3 is both constitutively activated (Mosser et al., 1992; Diamond et al., 1993; Hondalus et al., 1993), and expressed in a manner that exposes epitopes that bind an endogenous *M. tb.* ligand tighter than the competing C3. The exact conformation of the recombinantly expressed receptor, as well as the putative *M.tb.* surface ligand, still require further investigation.

The putative *M.tb.* CR3-binding surface ligand may not necessarily be a protein, as lectin-like properties have been attributed to CR3, as in the binding of various saccharides (Ross et al., 1985). Of great interest is the finding that *M.tb.* binds to CR3 at a site distinct from the C3bi-binding epitope, which is consistent with reports on nonopsonic binding of *M.tb.* in mouse macrophages (Stokes et al., 1993).

We therefore conclude that the heterologous expression of CR3 alone on the surface of non-phagocytic CHO cells is sufficient to confer phagocytic capabilities on this fibroblast cell line, to an extent greater than its inherent ability. Our results support the Isberg (1991) proposal, suggesting that integrins and other receptors are capable of internalising surface bound particles, providing that the adhesion is sufficiently strong.

In summary, *M.tb.* binds to CR3 in a complement-independent manner to a site distinct from the C3bi-binding site. Binding is sufficiently tight to promote uptake into a non-phagocytic cell transfected with CR3. This predicts that *M.tb.* expresses a high-affinity ligand for CR3 on its surface. If identified, such a ligand could be a useful target for the design of novel preventive and therapeutic strategies in tuberculosis.

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ADDENDUM

STANDARD METHODS, BUFFERS AND MEDIA

A.1 Standard Methods:

A.1.1 Large-scale isolation of *E. coli* plasmid DNA:

The preparation was based on the method of Ish-Horowitz and Burke (1981). Five-hundred millilitres of Luria broth (Addendum A.2) containing the appropriate selective pressure and was inoculated with *E. coli* grown overnight at 37°C, with good aeration at 200 rpm. Cells were pelleted by centrifugation at 5000 rpm for 10 minutes at 4°C, and resuspended in 10 ml of cold TEG (Addendum A.2) and 10 ml of 2 mg/ml lysozyme per 500 ml sample. Cells were incubated on ice for 30 minutes, with occasional agitation, before adding 40 ml of fresh, 0.2 M NaOH, 1% SDS per 500 ml sample and returning to ice for 10 minutes. Thirty millilitres of KAc (Addendum A.2) was added per sample and incubated for a further 30 minutes on ice, before centrifugation at 6000 rpm for 20 minutes. The supernatant was decanted into a clean centrifuge bottle, before precipitating the DNA by adding 1 volume of Isopropanol and incubating for 15 minutes at room temperature. The DNA was pelleted by centrifugation at 7000 rpm for 20 minutes and dissolved in 6 ml sterile, distilled water and 7 ml 5M LiCl and 0.05M MOPS, pH 8.0. The DNA was incubated on ice for 15 minutes before centrifugation at 60 000 rpm for 20 minutes at 4°C. The supernatant was again decanted and extracted with 1 volume of chloroform/isoamyl alcohol [24:1],

before pelleting by centrifugation at 6000 rpm for 20 minutes. The precipitated pellet was then washed with 5 ml of 70% cold ethanol and left to airdry overnight. The pellet was redissolved in 2.4 ml TE (Addendum A.2) and 100 µg/ml RNase A was added before incubation at 37°C for 15 minutes. The dissolved plasmid DNA was further purified by isopycnic CsCl-EthBr ultracentrifugation (Addendum A.1.3).

A.1.2. Small-scale isolation of *E. coli* plasmid DNA:

Plasmid DNA was extracted by an adapted Ish-Horowicz method. One-and-a-half millilitres of a 5 ml overnight culture of *E.coli*, grown at 37°C with shaking in LB broth with appropriate selective pressure, was pelleted in an Eppendorf microfuge for 3 minutes. The supernatant was aspirated and discarded and the pellet resuspended in 50 µl of TE buffer (Addendum A.2). One-hundred microlitres of cold phenol/chloroform was added to each resuspended pellet and vortexed for 2-3 minutes at room temperature. The Eppendorfs were centrifuged in the microfuge for 5 minutes at room temperature, to separate the proteins from the suspended DNA. Fifty microlitres of the top layer was carefully aspirated and transferred to a clean Eppendorf, before adding 18 µl of 7.5M NH_4Ac and 140 µl ice-cold ethanol and incubating at -20°C for 15 minutes, to precipitate the DNA. The DNA was pelleted by centrifugation in a microfuge for 15 minutes and washed with cold 75% ethanol, before being resuspended in 20 µl of TE and 1 µl of 10 mg/ml RNase A.

In the case of *E. coli* strains that contain active exo- and endonucleases, the following steps were added to the isolation procedure.

A sample of 20 µl of isolated DNA was removed and incubated together with 5 µl of 10% SDS, 0.5 µl of 10 mg/ml Proteinase K, and 76.5 µl of TE, to make up a final volume of 100 µl, at 65°C for 1 hour. The sample was further purified by using a Qiaex or Talent DNA purification kit as described in the package insert.

A.1.3. Plasmid purification by isopycnic CsCl-EthBr density gradient ultracentrifugation:

Plasmid purification by this means was performed as described by Maniatis et al. (1982). The crude plasmid extracts, as described in Addendum A.1.1., were resuspended in TE containing 1 g/ml final concentration of CsCl, and 400 µg/ml EthBr. The samples were decanted into Beckman Quickseal ultracentrifuge tubes, and sealed as instructed. Samples were centrifuged at 55 000 rpm for 18 hours at 15°C in a Beckman T.70i rotor, with the brake off. The plasmid bands were illuminated with ultraviolet light (350 nm) and the lower, covalently closed, circular plasmid band was aspirated with a syringe and a wide-gauge needle (approximately 3 ml DNA). To remove the contaminating EthBr, the samples were diluted with 2 volumes of 90% 1-Butanol with 1% SDS, vortexed briefly and centrifuged to separate out the EthBr in the top phase. This phase was removed and the procedure repeated until no trace of EthBr remained. The DNA sample was further purified to remove any contaminating salts by dialysis against 1 litre of TE, pH 8.0 for 48 hours at 4°C, with one buffer change. The purified DNA was then precipitated by adding 0.5 volumes of 5M NH_4Ac and 2 volumes of cold 100% ethanol and incubating at -70°C for 1 hour,

followed by centrifugation in a microfuge for 20 minutes. The pellet was further washed with 100 µl of cold 75% ethanol and the pellet left to airdry, before being dissolved in 100 µl of TE. The concentration of nucleic acids was determined spectrophotometrically by absorbance at 260 nm.

A.1.4. Restriction endonuclease digestion and DNA ligation reactions:

The methods are an adaptation of those described by Maniatis et al. (1982). Restriction endonuclease digests were performed in a total volume of 20 µl per reaction, with a restriction enzyme concentration of 3 units/µg DNA. Restriction enzyme buffers as supplied by the manufacturers were used (Boehringer Manneheim and Stratagene). Reactions were incubated at the suggested temperatures for between 1 and 3 hours.

Ligation reactions were all performed at room temperature for 9-16 hours in a total volume of 20 µl containing the supplied ligation buffer and 1 or 2 units of T4 DNA ligase (Boehringer Mannheim) per µg of purified DNA for sticky and blunt-end reactions, respectively. The total concentration of plasmid DNA present in vector-insert ligations was 10 to 15 pmole and 1 pmole for single-cut ligations. The vector and insert DNA ratios were at a molar ratio of 1:2 per ligation reaction. To control for ligase activity, a sample of single-cut plasmid DNA was included as a control.

A.1.5. Klenow reaction:

Previously digested and cleaned DNA suspended in TE was used in these reactions. To approximately 1 µg of DNA was added 2 units of Klenow (Boehringer Manneheim) and 1.5 µl of 10x reaction buffer M, as well as 2.25 µl of 1.25 mM dNTP's. The 15-µl reaction volume was incubated at 37°C for 15 minutes to enhance filling-in of the overhang, and then the Klenow was heat-inactivated at 65°C for 20 minutes. The sample was cleaned once again with either Qiaex or Talent Kits, to remove any contaminating dNTP's.

A.1.6. Preparation and transformation of competent *E. coli* cells:

A modified method of Cohen et al. (1972) and Dagert and Ehrlich (1979), was used for the preparation of competent *E. coli* cells. One viable colony of *E. coli* was picked from a fresh agar plate and inoculated into 10 ml of LB (Addendum A.2) and incubated overnight at 37°C with good aeration. The following morning, 2 ml of the overnight culture was inoculated into 100 ml of prewarmed LB and incubated at 37°C with shaking for approximately 2 hours, until a reading of 0.2 was reached at OD_{600}. The culture was then incubated on ice slush for 5 minutes, and all subsequent procedures were performed at 4°C to maintain cell integrity. Cells were harvested by centrifugation at 2000 x g for 5 minutes, followed by a wash in 10 ml cold 0.1M $MgCl_2$ and recentrifugation at 2000 x g for 5 minutes. The pelleted cells were washed in 10 ml of cold 0.1M $CaCl_2$ and incubated on ice for 30 minutes, before repelleting at

2000 x g for 5 minutes and resuspension in 2 ml of 0.1M $CaCl_2$. Cells were allowed to mature on ice for approximately 3 hours.

After the cells had aged, 50-200 ng of plasmid DNA was added to an aliquot of 100 µl of competent cells and kept on ice for approximately 10 minutes. The cell mixture was then heat-shocked at 42°C for 5 minutes and then quickly returned onto ice for 2 minutes, before adding 400 µl of LB to each aliquot in preparation for a 45-60 minute expression incubation at 37°C. Once the selective antibiotic expression was complete, a 100µl sample was plated onto LB agar plates containing the selective antibiotic. To control for spurious results and to monitor transformation efficiency, the following samples were included: competent cells with no added plasmid DNA or with uncut plasmid DNA. A transformation efficiency of approximately 5000 colonies/ng of plasmid-transformed cells was routinely observed.

A.1.7. DNA agarose gel electrophoresis:

Horizontal gel electrophoresis gel systems were used to separate DNA fragments. Agarose concentrations of between 0.8 and 2% were used together with TBE/EthBr buffer (Addendum A.2), depending on the DNA fragment sizes. Samples were loaded into agarose gel wells with a 25% final volume of DNA sample loading buffer (Addendum A.2). DNA bands were visualised with the aid of an ultraviolet (254 nm) Transilluminator and photographed using a Polaroid camera and Polaroid 667 film. The DNA fragments were sized by comparison to a standard curve obtained by the log molecular mass of known DNA fragment sizes versus their relative mobilities.

Standard DNA fragments were obtained by digesting λ DNA with *Hind III, Pst I, Eco RI* or *Pvu II.*

A.1.8. SDS-Polyacrylamide gel electrophoresis:

Laemmli (1970) and O'Farrell (1975) SDS-polyacrylamide gels (6%) methods were used with Biorad minigel apparatus. Acrylamide gel mixes were prepared as described in Addendum A.2. The gel mix was filtered through a bottle-top filter and the mixture retained on ice. A fresh 600µl aliquot of 10% ammonium persulphate (0.1g/ml) was added to 100 ml of gel mix, together with 65 µl of cold TEMED. Glass sequencing plates that had been clamped together, to provide a 0.2 mm internal space, were filled with the gel mix and allowed to set overnight at room temperature.

A.1.9. DNA sequencing:

Double-stranded DNA sequencing by the dideoxy xhain-termination method was performed as described in the Sequenase DNA sequencing manual (US Biochemical Corporation, Cleveland, Ohio). DNA sequencing templates were prepared as described in Addendum A.1.3. The 3-5µg of supercoiled, double stranded DNA was denatured by boiling for 3 minutes and placing on dry ice for 30 seconds, before preincubating with 12 ng of T3 or T7 primer on dry ice. Two microlitres of supplied reaction buffer were added and the tubes vortexed and centrifuged briefly to ensure contact of all components, before incubating at 37°C for 15 minutes. DNA chains

were radiolabelled with [^{35}S]dATP (Amersham). Sequencing gels were resolved on 0.2 mm 6% acrylamide gels as described in Addendum A.1.7. Gels were exposed on Kodak XAR-5 X-ray film for 24 hours to 5 days at -70°C.

A.1.10. Transfection or eukaryotic cells by the calcium phosphate precipitate method:

Cells were transfected in a manner similar to that described in Current Protocols in Immunology (1992). Exponentially growing CHO cells (Addendum A.1.11) were split and seeded at 1 x 10^6 cells/dish into 10 cm tissue culture dishes on the day prior to transfection. Cells were refed with complete media 3 hours prior to transfection, while 10-30 µg CsCl-purified plasmid DNA (Addendum A.1.3.) was ethanol-precipitated and redissolved in 450 µl of sterile water and 50 µl of 2.5M $CaCl_2$. Five-hundred millilitres of 2 x HeBS (Addendum A.2) was placed in a sterile 15 ml tube, and then the DNA/$CaCl_2$ solution was slowly added drop by drop and bubbled through with air. The DNA solution was allowed to incubate for 20 minutes at room temperature to produce a fine precipitate, which was added slowly to the 10 cm dish of adherent CHO cells. The cells were incubated with the precipitate at 37°C, 5% CO_2 for 4 hours, after which the precipitate was removed and the cells were glycerol-shocked with 2 ml of sterile 10% glycerol for 3 minutes at room temperature, to enhance transfection efficiency. The cells were washed with 5 ml of PBS 3 times and refed with complete media (Addendum A.2).

A.1.11. Culturing of CHO cell lines and hybridomas:

CHO cell lines were obtained as either frozen stocks or actively growing cells. Actively growing cells were pelleted by centrifugation at 1600 rpm for 5 minutes and resuspended in 1 ml of PBS, before being split 1:10 or 1:15 into fresh complete media (Addendum A.2) and seeded into tissue culture flasks. Cells were grown at 37°C with 5% CO_2 until confluent. Media were changed every 2-3 days.

Hybridomas were cultured as suspended cells in complete media (Addendum A.2). Confluent tissue culture flasks were decanted into 10ml tubes and the cells pelleted by centrifugation at 1500 rpm for 5 minutes. The cells were resuspended and split 1:5 into fresh media and a new tissue culture flask. Cells were grown at 37°C with 5% CO_2 and split every 3 days.

A.1.12. Freezing and thawing of cell lines:

Freezing:

Confluent, exponentially growing cells were lifted from the tissue culture flasks with 2 ml of 5mM EDTA/HBSS and washed by pelleting the cells gently in PBS. The pellet was then resuspended gently in 1 ml ice-cold Fetal Calf Serum (Gibco) with 10% DMSO (BDH). Point-five millilitre aliquots of cells were decanted into prelabelled Cryotubes (Nunc), tissue wrapped and placed at -70°C for two to three days before placing in a labelled freezer box at -70°C or transferring to liquid nitrogen canisters for long-term storage.

Thawing:

Aliquots of cells were removed from the -70°C freezer or liquid nitrogen and immediately thawed, with agitation in a 37°C water bath. The cells were then gently aspirated from the cryotube and placed into a sterile 10 ml tube and topped up with PBS. The cells were gently pelleted at 1000 rpm for 5 minutes, to remove the contaminating DMSO and resuspended in media containing 20% FCS, before seeding into tissue culture flasks and grown overnight at 37°C with 5% CO_2. The next day, the medium was removed and the cells reincubated with complete media.

A.1.13. Harvesting of hybridoma supernatant:

Actively growing cells were allowed to multiply until they started dying. The cells were spun down and discarded, and a fresh aliquot of actively growing cells was reincubated with the removed media. These cells were then grown in the media until their death, after which the cells were spun down and discarded. The supernatant was further purified of cell debris by centrifugation at 7000 rpm for 10 minutes. The culture supernatant was buffered with 1:20 volume of 1M Tris, pH 8.0, and 0.02% sodium azide added before storage at -20°C. If the supernatant was to be used in *in vivo* assays the supernatant may be filter-sterilised after buffering and then stored. With these harvesting methods a supernatant containing 20-50 µg/ml of antibody was expected.

A.1.14. Cell counting by haemocytometer:

Cells were pelleted and resuspended in 1 ml of PBS. A 10µl aliquot of cells was removed and resuspended with 90 µl of Turk's solution (Addendum A.2), providing a 1:10 dilution of cells. The cells were then injected underneath the weighted coverslip with a P200 tip and counted under a light microscope.

A.1.15. Ziehl-Neelsen staining of *M.tb.*:

M.tb. were harvested and an aliquot airdried onto a glass slide. Once the bacteria had dried onto the slide, they were heat-fixed by passing over a flame. The heat-fixed bacteria were then flooded with carbol fuchsin (Addendum A.2) and heated until steaming, three times. The carbol fuschin was washed off with water and the glass slides washed with copious amounts of acid alcohol (Addendum A.2), to remove all trace of stain. The slides were rinsed with water and counterstained with methylene blue (Addendum A.2) for 5 minutes. The slides were washed with running water and blotted dry before inspection under light microscopy, using 60x or 100x oil-immersion objectives.

A.1.16. Single-cell suspensions of *M.tb.*:

To obtain more or less single-cell suspensions of bacteria, 1 ml aliquots of the bacteria in Kirchner's medium (Addendum A.2) were transferred into 10 ml plastic tubes. The

bacteria were drawn up and forced through a series of syringe needles, as indicated below, to break up the clumps. Syringing proceeded from the larger to the smaller gauge needle, whenever the bacteria were flowing freely through the larger needle. All samples were ZN stained (Addendum A.1.14.), to check clumping and shearing, and grown to check viability.

1.	Orange needle	1.8 x 40 mm	
2.	Pink needle	1.2 x 38 mm	(18G)
3.	Green needle	0.8 x 40 mm	(21G)
4.	Black needle	0.7 x 32 mm	
5.	Tuberculin	0.5 x 16 mm	(25G)
6.	Brown needle	0.45 x 13 mm	(26G)
7.	Insulin	0.36x 12.7 mm	

A.1.17. Calibration curves for OD quantitation of *M.tb.*:

To attempt to and quantitate the number of bacteria present in a culture, a 3ml aliquot of various dilutions of *M.tb.*, as compared to McFarland's standards (Addendum A.2), was placed in a glass cuvette tube and read on a MSE spectro-plus spectrophotometer at 550 nm, previously blanked on 3 ml of fresh Kirchner's medium (Addendum A.2). The readings were compared to McFarland's standards and plated for colony counts to validate the results.

Number of Bacteria	A_{550}
6×10^8 organisms/ml	0.408
3×10^8 organisms/ml	0.202
1.5×10^8 organisms/ml	0.104
1.5×10^7 organisms/ml	0.011
1.5×10^6 organisms/ml	0.005

A.2. Buffers and Media:

Acid alcohol:

Ethanol	97 ml
HCl	3 ml

Acrylamide solution:

Urea	42 g
Acrylamide	5.7 g
Bis-acrylamide	0.3 g
TBE 10x	10 ml
Distilled water	50 ml

Stir on mild heat until dissolved and bring up to 100 ml volume. Filter gel mix through bottle top filter and keep on ice.

Cacodylate buffer (pH 7.4):

Cacodylate	0.1 M
$MgCl_2$	5 mM
$CaCl_2$	5mM

For fixation of eukaryotic cells, 0.1 M sucrose is added to the buffer.

Carbolfuchsin stain:

Basic fuchsin	0.3 g
Ethanol 95%	10 ml
Phenol	5 ml
Water	95 ml

Dissolve fuchsin in ethanol and then resuspend phenol in water. Mix together and allow to stand for several days. Filter before using.

DNA sample loading buffer:

Bromophenol blue	0.25% (w/v)
Glycerol	50% (v/v)
EDTA	100 mM
	(of final vol)

Heat-inactivated FBS:

Serum was heated to 56°C for 30 minutes.

KAc Solution:

Potassium acetate	29.44g
Glacial acetic acid	11.5 ml
Distilled water	88.5 ml

Kirchner's Agar:

Kirchner's media containing 1.5% agar.

Kirchner's Media (pH 7.0-7.3):

NaH_2PO_4	3 g
KH_2PO_4	4 g
$MgSO_4H_2O$	6 g
Na_3-citrate	2.5 g
Asparagine	5 g
Glycerol	20 ml
Distilled water	1000 ml

Loewenstein Jensen Slopes:

$MgSO_4.7H_2O$	0.24 g
Magnesium citrate	0.6 g
KPO_4	2.4 g
Asparagine	3.6 g
Glycerol	12 ml
Distilled water	600 ml
Potato flour	30 g
Homogenised whole eggs	1000 ml
Malachite green (2% aqueous)	20 ml

Add potato flour to salts and water, autoclave at 121°C for 30 minutes. Add homogenised eggs to cooled autoclaved solution, together with malachite green solution and aliquot. Inspissate the bottles by incubating at 85°C for 50 minutes. Store at 4°C for 1 month.

Luria Agar:

Luria Broth containing 1.5% agar.

Luria Broth:

Tryptone	10 g
Yeast	5 g
NaCl	10 g

Distilled water	1000 ml

Media was autoclaved at 15 lb./sq. inch for 20 minutes.

McFarland's standards:

Standard	Number of organisms/ml
0.5	1.5×10^8
1	3×10^8
2	6×10^8
3	9×10^8
4	12×10^8
5	15×10^8
6	18×10^8
7	21×10^8
8	24×10^8
9	27×10^8
10	30×10^8

Methylene blue counterstain:

Methylene blue chloride	0.3 g
Water	100 ml

PBS (pH 7.4):

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.44 g
KH_2PO_4	0.24 g
Sterile water	800 ml

RNase A:

RNase A (pancreatic)	50 mg
NaCl (1 M)	75 µl
Tris HCl pH 7.5 (1 M)	50 µl
Distilled water	< 5 ml

Dissolve above in water and boil for 15 minutes. Allow to reach room temperature and store at -20°C.

TBE buffer (pH 8.0):

Tris HCl	89 mM
Boric acid	89 mM
EDTA	2.5 mM

TE Buffer (pH 8.0):

Tris HCl	10 mM
EDTA	1 mM

TE-equilibrated phenol:

0.1% final concentration of 8-hydroxyquinoline was added to 100 ml melted phenol. The mixture was equilibrated with two volumes of 1.0-M Tris, pH 8.0 and two volumes 0.1-M Tris, pH 8.0 with 0.2% ß-mercaptoethanol, by mixing the phases and allowing them to separate, repeatedly. The equilibrated phenol was stored under 0.1M TE buffer in the dark, at -20°C.

TEG Buffer (pH 8.0):

Glucose	50 mM
Tris	25 mM
EDTA	10 mM

Tissue culture complete media:

CHO-WT complete media:

DMEM (Highveld Biological)	450 ml
Ham's F12 (Highveld Biological)	450 ml
FBS (Gibco), heat inactivated	100 ml

CHO-MAC-1 complete media:

α-MEM (Gibco)	850 ml
Dialysed FBS (Gibco)	100 ml
Thymidine 1.6 mM (Sigma)	10 ml
Methotrexate 2 μM (Sigma)	5 ml

CHO-CR3 complete media:

GMEM (Gibco)	850 ml
Dialysed FBS (Gibco)	100 ml
Nonessential amino acids 100x (Gibco)	10 ml
Glutamate & Asparagine (Sigma)	10 ml
Sodium pyruvate 100 mM (Gibco)	10 ml
Nucleoside mix 50x (Sigma)	20 ml

Nucleoside mix: 35 mg of each:adenosine, guanosine, cytidine, uridine and thymidine (Sigma)

Made up with 100 ml sterile water and filtered.

Glutamate and Asparagine: 600 mg of both L-glutamate and L-asparagine, made up with 100 ml of sterile water.

Macrophage media:

RPMI-1640 (Highveld Biological)	90 ml
Human serum (autologus)	10 ml

Hybridoma media:

RPMI-1640 (Highveld Biological)	79 ml
L-glutamine (200 mM)	1.2 ml
FBS (Gibco), heat-inactivated	20 ml

Turk's Stain:

Gentian violet	0.02 g
Glacial acetic acid	7.14 ml
Distilled water	100 ml