CLONING OF THE DNA OF *MYCOBACTERIUM TUBERCULOSIS* AND ANALYSIS OF THE EXPRESSED PROTEIN ANTIGENS

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ABSTRACT

Mycobacterium tuberculosis genomic DNA fragments with randomly generated endpoints were used to construct a recombinant λ gt 11 DNA expression library. This library was screened using polyclonal anti-M.tuberculosis serum, and anti-β-galactosidase monoclonal antibodies. Ten recombinant phage clones showing reactivity to these antibodies were isolated. Restriction enzyme analysis of these clones revealed that the clones contained DNA inserts ranging between 1.9 kilobases and 8.0 kilobases in size. Stable lysogens were generated from nine of the recombinant clones, and the recombinant proteins produced by the lysogens were analysed by polyacrylamide gel electrophoresis and Western blotting. Each of the recombinant clones was found to produce recombinant non-fusion proteins 32 kilodaltons and 50 kilodaltons in size. In addition, β-galactodidase fusion proteins of molecular weights 22 kilodaltons, 12 kilodaltons, and 6 kilodaltons were produced by the recombinant phages. Preliminary studies indicate that the 32 kilodalton recombinant antigen is capable of inducing lymphoproliferative responses in mononuclear cells from tuberculous patients.

I declare that the dissertation entitled " Cloning of the DNA of Mycobacterium tuberculosis and analysis of the expressed protein antigens" presented for the Degree of Master of Science at the University of the Witwatersrand, Johannesburg, is my own unaided work, and has not been presented for any degree or examination at any other university. Permission to work with rabbits was obtained from the Animal Ethics Committee, University of the Witwatersrand, Johannesburg (Clearance Certificate Number 88 162 3).

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Presentations and publications

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DEDICATION

For my family, for their love, support, and constant encouragement.

And for Edgar Allan Poe ...

" Science! true daughter of Old Time thou art!
Who alterest all things with thy peering eyes.
Why preyest thou thus upon the poet's heart,
Vulture, whose wings are dull realities?
How should he love thee? or how deem thee wise,
Who wouldst not leave him in his wandering
To seek for treasure in the jewelled skies,
Albeit he soared with an undaunted wing?
Hast thou not dragged Diana from her car?
And driven the Hamadryad from the wood
To seek a shelter in some happier star?
Hast thou not torn the Naiad from her flood,
The Elfin from the green grass, and from me
The summer dream beneath the tamarind tree? "

Sonnet - to Science
Edgar Allan Poe (1829)

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LIST OF ABBREVIATIONS

Note: Formulae for chemical compounds are not included in this list.

A	amperes
ADA	adenosine deaminase
AIDS	acquired immunodeficiency syndrome
ATP	adenosine triphosphate
β	beta
BCG	Bacillus Calmette-Guerin
bp	base pairs
BSA	bovine serum albumin
^{o}C	degrees celcius
cDNA	complementary DNA
Ci	Curie
CIE	crossed immunoelectrophoresis
cm	centimeter
cpm	counts per minute
CSF	cerebrospinal fluid
dH_2O	distilled water
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
γ	gamma
g	gravity

HLA	human leukocyte antigen
i.e.	that is
IFN-γ	interferon gamma
IgG	immunoglobulin G
IL 1	interleukin 1
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton
λ	lambda
M	molar
mA	milliampere
mg	milligram
MHC	major histocompatibility complex
ml	millilitre
mm	millimeter
mM	millimolar
mRNA	messenger RNA
ng	nanogram
nm	nanometer
no.	number
O.D.	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate bufferred saline
pfu	plaque forming unit
PGE	prostaglandin E
PPD	purified protein derivative

Rf	relative motility
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TBS	Tris bufferred saline
TNF	tumour necrosis factor
U	unit (enzyme unit)
uCi	microcurie
ug	microgram
ul	microlitre
um	micrometer
uM	micromolar
V	volt
viz.	namely
Xgal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Tuberculosis is a chronic, granulomatous infection of man that is caused by Mycobacterium tuberculosis (M.tuberculosis). Tuberculosis is found worldwide, although Third World countries are most seriously affected with the disease. Mycobacterial infections in the Western world are however on the increase, and vaccination and chemotherapy are failing to eliminate them (Lancet Editorial, 1980). The emergence of the acquired immunodeficiency syndrome (AIDS) has also focused attention on the sudden rise in the incidence of mycobacterial disease in the Western world (Raymond, 1986). There are estimated to be between 20 and 30 million new cases of tuberculosis, and 3 million deaths from tuberculosis, per year. Tuberculosis therefore ranks as the leading cause of death resulting from an identifiable infectious agent (Ellner, 1986). Although much progress has been made in the characterisation of mycobacterial antigens, and knowledge of their immunological importance has increased, extensive research is still needed to rid the world of a disease which "ought to be a disease of antiquity", and for which "every single case ought to be viewed as a failure in public health" (Raymond, 1986).

1.2 TRANSMISSION AND RESERVOIR OF INFECTION

Tuberculosis is primarily a disease of humans. Man is the major reservoir of infection, and the disease is transmitted by direct person-to-person aerosol transmission of droplets formed when infectious material is discharged into the air by coughing or other means (Youmans, 1986). The portal of entry of the infection is usually the lower respiratory tract, and the major site of the initial infection is the lung (Youmans, 1986). Tuberculosis can also be acquired by way of the gastrointestinal tract. In this case, however, the pathogenic organism is Mycobacterium bovis (M.bovis), which causes a pulmonary disease that is indistinguishable from tuberculosis (Youmans, 1986). This microorganism may be found in large numbers in the milk of infected cows, and ingestion of such milk by humans may result in extensive tuberculous disease. However, effective control of tuberculosis in cattle and the pasteurisation of milk have virtually eliminated tuberculosis in humans caused by M.bovis (Youmans, 1986).

1.3 PATHOGENESIS OF TUBERCULOSIS

The first major manifestations of the disease occur in the lung, from where it can disseminate to almost any tissue and organ in the body (Grange, 1980). Multiplication of M.tuberculosis occurs most rapidly in the lung, possibly due to the high concentration of molecular oxygen, which the

mycobacteria require for growth (Youmans, 1986). The progress of infection then takes one of two courses, depending on whether the infection is primary, or whether it occurs in a person previously infected with tuberculosis (Grange, 1980). If the infection is primary, mycobacteria lodge within the alveolus, where they are rapidly phagocytosed by alveolar macrophages (Grange, 1980). The mycobacteria are resistant to destruction in these macrophages, for reasons not yet wholly clarified, but which include the inhibition of fusion between the phagosomes and lysosomes inside the macrophages (Wadee, Cohen, and Rabson, 1987). The rate of replication of the tubercle bacilli inside the macrophages is slow, and therefore the appearance of symptoms or pathological conditions due to a tuberculous infection may require several weeks. When the number of tubercle bacilli becomes significant, an inflammatory cellular exudate appears (Youmans, 1986). Dissemination from the primary focus of infection now occurs within a few hours, primarily by way of the lymphatic system. At the same time, spillover from the lymphatics to the bloodstream occurs, resulting in a seeding of virulent bacilli to all tissues and organs of the body (Youmans, 1986). This process may advance until widespread tuberculous disease, and possibly death, occurs (Youmans, 1986). In the majority of primary infections, however, after a period of a few weeks the rate of multiplication of the bacilli markedly decreases, possibly as a result of activation of the infected macrophages (Collins, 1988).

These cells now demonstrate a number of biochemical and physiological characteristics which differentiate them from normal macrophages, including an increased number of mitochondri , lysosomes, increased metabolic activity, spreading, phagocytosis, and increased tuberculocidal a tivity in the form of released hydrolytic enzymes and hydrogen peroxide (Wadee, 1985; Armstrong and D'Arcy Hart, 1971; Hahn and Kaufmann, 1981). The pneumonic process now resolves and dissemination to other organs ceases.

Coincident with these changes, two immunological manifestations appear (Chaparas, 1982): first, the macrophages in which the bacilli were initially able to replicate now non-specifically express antimicrobial and enhanced tumoricidal activity; second, the infected individual shows a cutaneous reaction of delayed hypersensitivity to certain proteins and polypeptides (tuberculin) found in the tubercle bacilli .

Tuberculin hypersensitivity is specific to tuberculoprotein or polypeptide. It does not involve circulating antibodies, and can be transferred passively using lymphocytes from tuberculin-sensitive animals (Collins, 1988). Such lymphocytes, in the presence of tuberculoprotein, elaborate the release of lymphokines such as interferon γ (IFN-γ) and tumour necrosis factor (TNF) (Rook, 1987), which may be responsible for the destructive inflammatory reactions

characteristic of tuberculosis.

Acquired cellular immunity to tuberculosis also does not involve mycobacteria-specific circulating antibodies, which are present but have been found to play no role in host protection (Reggiardo and Middlebrook, 1974). Specific resistance has been shown to be mediated by antigen-specific lymphocytes, with a minor role being played by activated macrophages (Hahn and Kauffman, 1981).

Until tuberculin hypersensitivity and acquired immunity develop, primary tuberculosis is for the most part a non-destructive disease. Resolution of the disease may proceed to a point where little or no residue of infection remains. Reinfection tuberculosis (secondary tuberculosis), however, is a disease which occurs in the presence of tuberculin hypersensitivity and in spite of specific cellular immunity (Youmans, 1986). Secondary infection may be endogenous, occurring as a result of recrudescence of a primary infection, or exogenous, occurring due to infection from an active case. Regardless of the source, the initial lesion is characterised by necrosis, as a result of the inflammatory reaction due to tuberculin hypersensitivity. The lesion is also localised by sensitised macrophages and lymphocytes which, found in the adjacent tissues and draining the lymphatics, prevent multiplication of the tubercle bacilli and spread of the disease (Grange, 1980).

However, although multiplication of the tubercle bacilli in cells of the immune system is inhibited, often not all of the bacilli are destroyed, and may remain viable but dormant within the tissues for many months or years (Youmans, 1986). The disease may then spread by extension, as adjacent tissues develop necrosis as a consequence of inflammation. Spread may also occur when a bronchus is eroded (bronchogenic spread), or may occur via the bloodstream if a blood vessel is eroded (haematogenous spread) (Youmans, 1986).

The reasons for the local breakdown of resistance that lead to a necrotic focus are not well understood. They are thought to include the presence of other degenerative diseases (Youmans, 1986), physical fitness, body build and state of nutrition (Snider, 1987), age (Orme, 1987), possible genetic predisposition (Mehra and Bovornkitti, 1986; Bahr, Rook, Shahin, et al., 1988; Al-Arif, Goldstein, Affronti, et al., 1979), and acquired or induced immunosuppression (Ellner, 1986).

1.3.1 The immunopathology of tuberculosis

Mycobacterial constituents and cytokines produced in response to mycobacterial infection have been shown to be largely responsible for the pathological reactions associated with mycobacterial infection (Rook, 1987). The mycobacterial

component muramyl dipeptide has been shown to cause severe haemorrhagic and necrotic inflammation at local sites of injection in guinea pigs (Nagao, Takada, Yagawa, et al., 1987). Potent macrophage primers, such as IFN-γ and vitamin D3 metabolites, (Rook, Steele, Fraher, et al., 1986), could also prime these cells to release tumour necrosis factor (TNF) (Rook, Taverne, Leveton, et al., 1987). TNF has been demonstrated to be pyrogenic, to cause weight loss (Beutler and Cerami, 1985), and to be a major mediator of toxic shock. Endothelial damage and fibrin deposition have also been shown to be characteristic of tuberculous lesions (Rook, 1987): TNF has also been shown to lead to the loss of normal anticoagulant properties of the vascular endothelium (Bevilacqua, Pober, Majeau, et al., 1986). Acting locally, the release of TNF could account for much of the tissue necrosis seen in tuberculosis (Rook, 1987).

1.4 THE IMMUNE SPECTRUM IN TUBERCULOSIS

1.4.1 T lymphocytes

T cells have been shown to play a mandatory role in acquired resistance to mycobacterial infection. The exact mechanisms of T cell interactions are however still poorly understood (Lamb and Rees, 1988). It is currently believed that sensitised T cells, on recognition of antigen in association with class II histocompatibility antigens, release

lymphokines such as macrophage activating factor or IFN-γ (Lowrie, 1983). These lymphokines activate or prime macrophages such that they are able to express enhanced antimicrobial activity against the infecting organism (Lowrie, 1983). Recent evidence has also indicated that T cells can directly kill macrophages infected with M.tuberculosis (Rook, 1987). Mycobacterial antigen-specific cytotoxic T cells which kill antigen-presenting macrophages have been defined as CD 3^+, CD 4^+, CD 8^-, and have been shown to be restricted by HLA class II antigens (Hansen, Petersen, Povlsen, et al., 1987; Mustafa and Godal, 1987). Sensitised T cells have also been shown to release cytokines that promote the migration of blood-borne monocytes to the site of mycobacterial infection, and which contribute to the formation of a granuloma (Chaparas, 1982). They have also been demonstrated to mediate hypersensitivity to mycobacterial infection, and to be responsible for immunologic memory (North, Mackaness, and Elliot, 1972; Orme, 1987). Passive transfer experiments have provided evidence that different populations of T cells play a protective role in mycobacterial infections: this may reflect switching from a state of active immunity to one of immunologic memory (Orme, 1987 b; Muller, Cobbold, Waldmann, et al., 1987; Boom, Husson, Young, et al., 1987).

Little is known about the antigens that are important in the generation of protective immunity and memory. Recent evidence has indicated that antigens from viable mycobacteria are required for the induction of specific protective immunity (Orme, 1988). Antigens from dead organisms were able to induce delayed hypersensitivity and mediate helper signals to antibody-producing B cells, but did not induce protective cellular immunity (Orme, 1988).

1.4.2 Macrophages

Even though it has been suggested that the effector mechanisms required for the host's bactericidal activity against mycobacteria reside primarily within the macrophage (Chaparas 1982), there are some doubts as to whether the activated macrophage is the final effector cell (Rook, 1987). However, normal macrophages have been shown to demonstrate limited bactericidal ability (Chaparas, 1982), and require priming or activation by T-cell-derived mediators (Ivanyi, 1986) for their bactericidal activity. The contribution of these mediators seems mandatory since macrophage tuberculocidal activity occurs only in the presence of such mediators (Ivanyi, 1986). Amongst these, interferon-γ (IFN-γ) has been shown to confer an enhanced intracellular killing ability to macrophages (Rook, Steele, Fraher, et al., 1986; Flesch and Kaufmann, 1987). In addition, IFN-γ has been demonstrated to be able to reverse the inhibition of

leukocyte bactericidal activity mediated by mycobacterial antigens (Wadee, Cohen, and Rabson, 1987 b).

1.4.3 Granulocytes

Polymorphonuclear leukocytes have been demonstrated to be able to mediate intracellular killing of mycobacteria (Brown, Holzer, and Andersen, 1987). Killing by neutrophils is enhanced when they are activated, and is mediated by reactive oxygen intermediates produced during the respiratory burst, as well as by non-oxygen-dependent mechanisms.

1.4.4 The humoral response

Antibodies do not confer passive protection to mycobacterial infection (Reggiardo and Middlebrook, 1974) and although the contribution of B cell responses to the pathogenesis of tuberculosis has not been ruled out (Ivanyi, 1986), the main interest in antibodies has been devoted to diagnostic purposes.

1.5 THE DIAGNOSIS OF MYCOBACTERIAL INFECTION

1.5.1 Isolation and identification of mycobacteria

The diagnosis of active tuberculosis can only be made if tubercle bacilli are isolated from lesions or affected organs. Since tuberculosis usually is a pulmonary infection, sputum is the specimen most commonly examined for the presence of tubercle bacilli (Youmans, 1986). Urine, spinal fluid or tissue biopsies are often also examined. Microscopic examination of specimens involves the identification of acid-fast bacilli (Grange, 1980), but this does not distinguish virulent tubercle bacilli from contaminating saprophytic bacteria or from atypical mycobacteria. The presence of acid-fast bacilli in a specimen, therefore, does not provide a definite diagnosis of tuberculosis. Isolation of virulent bacilli is performed by culturing specimens on specialised media such as Lowenstein-Jensen medium (Grange, 1980). However, owing to the slow multiplication rate of the tubercle bacilli, in most cases growth cannot be detected for 3-6 weeks.

1.5.2 Non-immunological diagnostic tests

Several non-immunological tests useful for the diagnosis of tuberculosis are available: measurement of adenosine deaminase (ADA) activity in pleural, peritoneal, pericardial

and cerebrospinal fluid (CSF) is used for the diagnosis and follow-up of tuberculous infection (Ribera, Martinez-Vasquez, Ocana, et al., 1987); the radioactive bromide partition test can be useful for the detection of tuberculous meningitis (Daniel, 1987); the biochemical identification of mycobacterial 3-(2'-ketohydroxyl)-indoline and tuberculostearic acid in the sputum and CSF can be diagnostic of tuberculous disease (Daniel, 1987; Drutz and Graybill, 1987).

Unfortunately, none of the above tests can be used to provide a conclusive diagnosis of tuberculosis, and the complex instrumentation necessary precludes these measurements being used in developing countries.

1.5.3 Serological tests for tuberculosis

Several serodiagnostic tests have been employed for the diagnosis of tuberculosis. These include fluorescent antibody tests (Affronti, 1985), complement fixation, agglutination, precipitation, and gel diffusion tests (Grange, 1980). These tests are however for the most part highly non-specific and non-sensitive, and have thus fallen aside.

Radioimmunoassay (Kadival, Samuel, Mazarello, et al., 1987; Ashtekar, Dhalla, Mazarello, et al., 1987) and enzyme-linked immunosorbent assay (ELISA) techniques (Wadee, Cohen, and