IMMUNOLOGICAL STUDIES OF DNA (pJWVacII) AND SURFACE DISPLAY (r-STVacII) VACCINE CANDIDATES EXPRESSING A SYNTHETIC MULTIEPITOPE GENE OF *MYCOBACTERIUM TUBERCULOSIS* IN A PRIME BOOST STRATEGY USING A MOUSE MODEL

by

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LIST OF ABREVIATIONS

AFB	Acid fast bacillus
αβ	Alpha beta
Ag85	Antigen 85
APCs	Antigen presenting cells
BCG	Bacille Calmette Guerin
CMI	Cell mediated immunity
CFU	Colony forming unit
CTL	Cytotoxic T lymphocyte
ddH_2O	Deionised distilled water
DTH	Delayed type hypersensitivity
DCs	Dendritic cells
DNA	Deoxyribonucleic acid
ER	Endoplasmic reticulum
γδ	Gamma delta
HIV	Human Immunodeficiency Virus
HLA	Human leukocyte antigen
IFN	Interferon
IL	Interleukin
i.m	Intramuscular
i.p	Intraperitoneal
kDa	kilodalton
LB	Luria-Bertani
MHC	Major histocompatibility complex
mAbs	Monoclonal antibodies
MDR-TB	Multi-drug resistant TB
NAA	Nucleic acid amplification
NK	Natural killer
Nramp	Natural-resistance-associated macrophage protein
O.D	Optical density
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction

PPD	Purified protein derivative
rBCG	Recombinant bacilli Calmette Guerin
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RD	Region of difference
RE	Restriction enzyme
SIV	Simian immunodeficiency virus
SI	Stimulation index
Th	T helper
TAP	Transporter associated protein
TB	Tuberculosis
TLR	Toll-like receptor
TNF	Tumor necrosis factor
UV	Ultraviolet
WHO	World Health Organization

KAJIAN IMUNOLOGI CALON VAKSIN DNA (pJWVacII) DAN VAKSIN 'SURFACE DISPLAY' (r-STVacII) YANG MENGEKSPRESKAN GEN MULTIEPITOP SINTETIK *MYCOBACTERUM TUBERCULOSIS* DALAM STRATEGI 'PRIME BOOST' MENGGUNAKAN MODEL MENCIT.

ABSTRAK

Tuberculosis (TB) pada manusia adalah disebabkan oleh patogen bakteria *Mycobacterium tuberculosis* dan merupakan salah satu penyakit utama di dunia. Satu-satunya vaksin TB yang terdapat pada masa ini ialah strain *M. bovis* yang telah dilemahkan iaitu, Bacille Calmette Guerin (BCG). Bagaimanapun, efikasi perlindungan BCG merangkumi julat 0 ke 80% di tempat kajian yang berlainan. Kesan perlindungan BCG adalah ketara pada kanak-kanak tetapi tidak menunjukkan perlindungan terhadap TB paru-paru di kalangan orang dewasa yang menjadi masalah utama. Vaksin DNA merupakan salah satu cara baru untuk mengawal penyakit berjangkit dan boleh merangsang tindak balas kedua-dua sel humoral dan selular. Hasil kajian lepas, calon vaksin pJWVacII dan r-STVacII telah digunakan dalam kajian ini dengan menggunakan strategi *'prime-boost'*. Vaksin DNA, pJWVacII telah diberikan secara intraotot kepada mencit manakala vaksin *'surface display'* pula telah diberikan secara oral kepada mencit. Splenosit dari mencit yang diimunisasi telah diuji dengan pelbagai ujian keimunan. Keputusan menunjukkan bahawa splenosit dari mencit yang diimunisasi memberikan peningkatan gerak balas proliferasi apabila dirangsang dengan antigen (Inak-nVacII). Analisis sitokin intrasel ke atas splenosit juga menunjukkan kedua-dua $CD4^+$ dan $CD8^+$ sel T menghasilkan IL-2 dan IFN-γ berikutan rangsangan antigen. Dalam kaedah 'prime-boost', kajian menunjukkan kaedah *'prime'* dengan pJWVacII dan *'boost'*

dengan r-STVacII adalah strategi terbaik untuk merangsang respon keimunan dalam mencit. Sebagai kesimpulan, data yang diperolehi dari kajian ini mencadangkan bahawa vaksin DNA digabungkan dengan vaksin 'surface display' menggunakan kaedah *'prime-boost'* merupakan salah satu strategi baru untuk membangunkan calon vaksin terhadap TB.

IMMUNOLOGICAL STUDIES OF DNA (pJWVacII) AND SURFACE DISPLAY (r-STVacII) VACCINE CANDIDATES EXPRESSING A SYNTHETIC MULTIEPITOPE GENE OF *MYCOBACTERIUM TUBERCULOSIS* IN A PRIME BOOST STRATEGY USING A MOUSE MODEL

ABSTRACT

Tuberculosis (TB) in humans is caused by the bacterial pathogen *Mycobacterium tuberculosis* and is still a major health problem worldwide. The only TB vaccine currently available is an attenuated strain of *M. bovis*; Bacille Calmette Guerin (BCG). BCG demonstrated variable protective efficacies ranging from 0 to 80% in different field trials. BCG is effective at preventing childhood manifestation of TB but it does not prevent the most prevalent disease which is pulmonary TB in adults. DNA vaccination is an important new approach to the control of infectious agents and induces both humoral and cellular immune responses. Two previously constructed vaccine candidates, pJWVacII and r-STVacII were used in this study employing a prime-boost strategy. The naked DNA vaccine, pJWVacII was given intramuscularly to mice whilst the surface display vaccine, r-STVacII was given orally. Splenocytes from the vaccinated mice were tested for various immunological tests. The results showed that splenocytes from immunized mice were found to proliferate more aggressively when stimulated with the antigen (Inak-nVacII). Flow cytometric intracellular cytokine analysis of splenocytes from vaccinated mice also showed that both $CD4^+$ and $CD8^+$ T cells produce IL-2 and IFN-γ following stimulation with the antigens. In the prime-boost approach, the study showed that mice primed with the naked DNA vaccine, pJWVacII and boosted with the surface

display vaccine, r-STVacII is the best strategy to stimulate immune response in mice. As a conclusion, the data obtained from this study suggest that DNA vaccination in combination with surface display vaccination using prime-boost approach provides a new strategy for developing a candidate vaccine against TB.

CHAPTER 1

INTRODUCTION

1.1 Introduction to TB

Tuberculosis (TB) is a contagious and potentially fatal disease that can affect almost any part of the body but manifests mainly as an infection of the lungs. It is caused by a bacterial microorganism, the tubercle bacillus or *Mycobacterium tuberculosis*. TB infection can either be acute and short-lived or chronic and long-term. Approximately 1.7 billion people or one-third of the world's population are infected with the predominant TB organism, *M. tuberculosis*. Currently, 10 to 15 million people in the United States have latent TB infection, and 10 percent of them will develop active disease at some point in their lives (Diana, 2000).

Most people infected with *M. tuberculosis* never develop active TB. However, in people with weakened immune systems, especially those infected with the human immunodeficiency virus (HIV), TB organisms may overcome the body's defenses, multiply, and cause active disease (Ellner, 1990). Each year, 8 million people worldwide develop active TB and 3 million die. TB is often one of the first secondary infections to be activated in HIV – positive individuals. Moreover, poverty, malnutrition and their contributing factors such as political disorganization and war also contribute to the increase rate of TB. Figure 1.1 shows TB notification rate around the world.

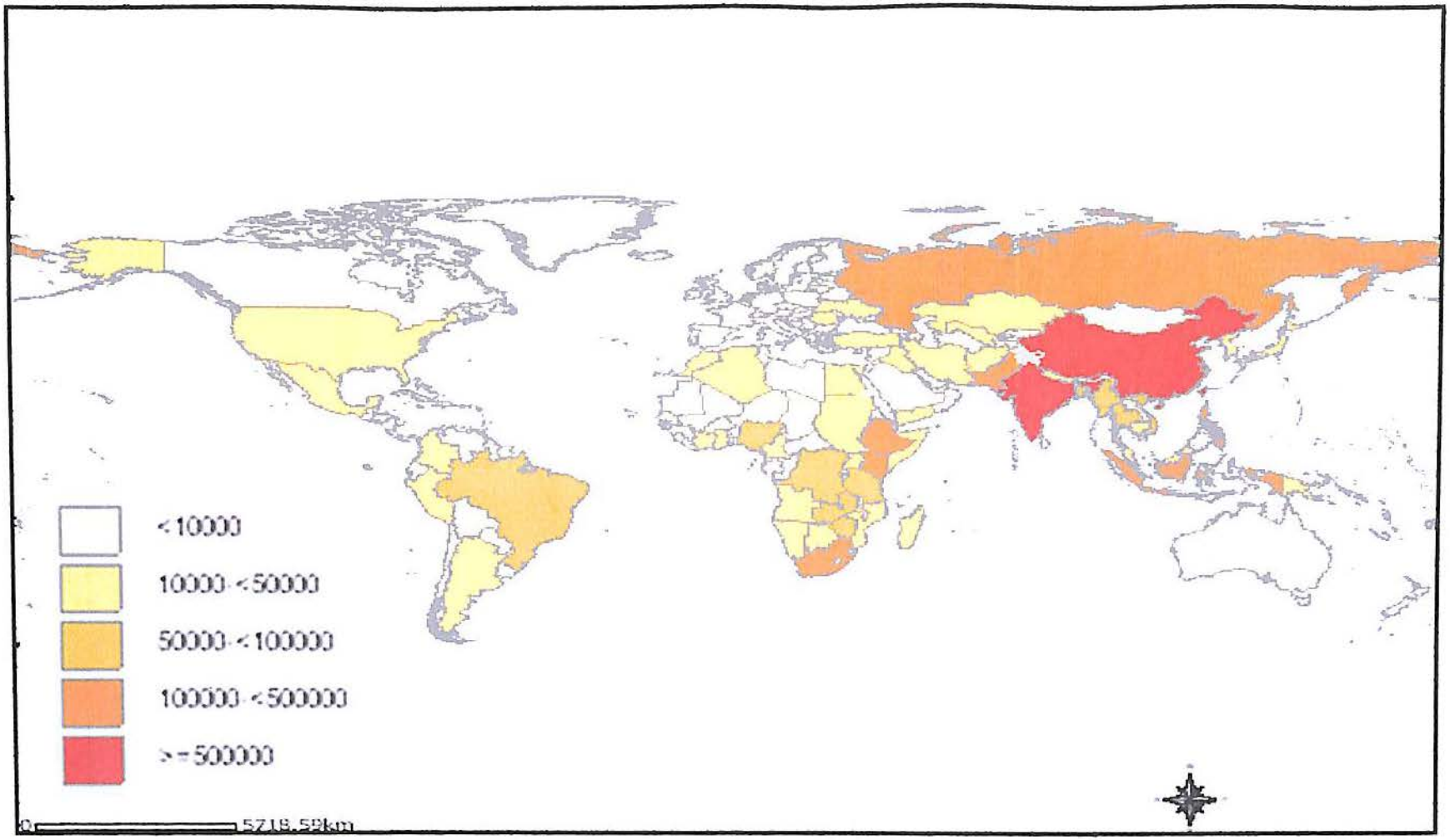

Figure 1.1: Tuberculosis notification rates, as of 22 March 2006 (Source: WHO Stop TB Department, website: www.who.int/tb)

1.2 History of TB

In the second half of the 17th century, when TB caused high levels of death rates in Europe, John Bunyan gave the title 'captain of all these men of death' for TB, which was also known as the white plaque. Pulmonary TB was known since the time of Hippocrates as phthisis, derived from the Greek for 'wasting away'. In 1680, the French, Franciscus Sylvius carried out anatomic-pathologic studies in pulmonary nodules from TB patients, which he named as 'tubercula' (small knots). These knots were believed to be only some type of tumor or abnormal gland. In 1722, a British doctor Benjamin Marten proposed that TB could be transmitted through the 'breath' of a sick person (reviewed by Rodrigo *et al.*, 2006).

In 1882, Robert Koch isolated and cultured *M. tuberculosis* from crushed tubercles and identified the bacterium as the TB etiological agent. In 1896, an American bacteriologist demonstrated that bovine TB was not caused by *M. tuberculosis* but by a closely related bacterium, *M. bovis*. Twelve years later, Albert Calmette and Camille Guerin isolated the bovine variant from its host and grew the bacilli in dispersed culture containing ox bile. After 13 years of experimentation, the variant was administered for the first time in humans orally, as an attempt to immunize a child whose mother died in childbirth as a victim of TB.

However, WHO indicated that there have not been great effects on the global problem since the time of Koch (Bloom & Murray, 1992). Currently, TB causes

more human deaths than any other single infectious agent, standing for 26% of all preventable deaths and 7% of all deaths (reviewed by Rodrigo *et al.*, 2006).

1.3 *Mycobacterium tuberculosis*

The TB bacterium is a rod-shaped bacterium, non-motile, non-spore forming, 1-4 μm in length, and between 0.3-0.6 μm in diameter, making them smaller than most bacterial pathogens (Iseman, 2000 and Akemi *et al.*, 2003). This bacterium belongs to the family Mycobacteriaceae and the order Actinomycetales. *M. tuberculosis*, like other mycobacteria, has an unusual cell wall, a waxy coat comprised of fatty molecules whose structure and function are not well known. This cell wall appears to allow *M. tuberculosis* to survive in its preferred environment: inside immune cells called macrophages, which ordinarily degrade pathogens with enzymes. The coat of *M. tuberculosis* also renders it impermeable to many common drugs.

M. tuberculosis and other mycobacteria are also called as "acid fast" bacteria (AFB) which means that they retain certain dyes following an acid-alcohol decolorization step and this characteristic is related to the complex cell wall structure that contains derivatives of mycolic acid (Floyd *et al.*, 1992). In the most common staining technique, Ziehl-Neelsen stain, AFB is stained a bright red which stands out clearly against a blue background. It can also be visualized by fluorescent microscopy and by auramine-rhodamine stain (Batzing, 2002).

There are several factors that contribute to the difficulty in the study of *M. tuberculosis* in the laboratory. First, the bacteria multiply very slowly, only once every 24 hours and take a month to form a colony. Two media are often used to grow *M. tuberculosis*; Middlebrook's medium which is an agar based medium and Lowenstein-Jensen medium which is an egg based medium (American Thoracic Society, 2000).

In comparison, other organism such as *E. coli* form colonies within eight hours. Moreover, TB bacilli tend to form clumps which are difficult to work with or to count the cells. Most daunting, *M. tuberculosis* is a dangerous, airborne organism that can be studied only in laboratories that have specialized safety equipment.

Several species of mycobacteria with similar growth characteristics and biochemical reactions are classified together as the *M. tuberculosis* complex (Cole, 2002). This complex includes *M. bovis, M. africanum* and *M. microti* which can also cause TB in mammals. The first two are very rare causes of disease and the last one do not cause human disease (Brosch *et al.*, 2000).

1.3.1 The cell wall of *M. tuberculosis*

Mycobacteria produce an extremely uncommon cell wall structure. It is composed of a multilayered cell envelope which basically consists of (from inside the cells to the outer surface): a plasma membrane and three covalently associated macromolecules such as peptidoglycan, arabinogalactan and mycolic acid or glycolipids (Figure 1.2). The plasma membrane is composed of

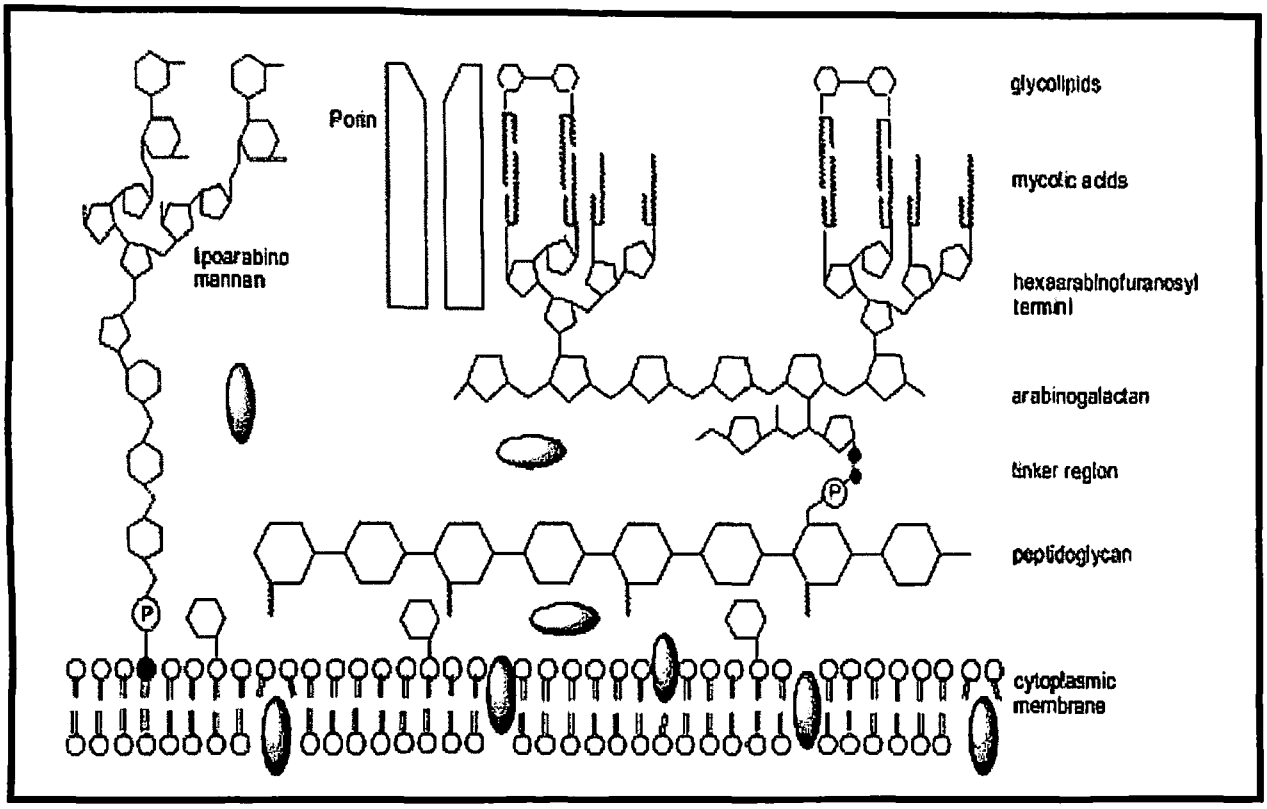

Figure 1.2: Schematic representation of the mycobacterial cell wall (Adapted from Rodrigo *et al.*, 2006)