Generation and characterization of DNA vaccine against anthrax enabling delivery of protective antigen (PA63) to appropriate processing pathways

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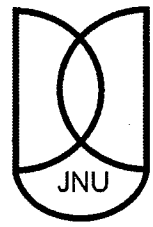
By

SHUCHI MIDHA



2008

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CERTIFICATE

This is to certify that the work entitled, **'Generation and characterization of DNA vaccine against anthrax enabling delivery of Protective Antigen (PA63) to appropriate processing pathways'** submitted to the School of Biotechnology, Jawaharlal Nehru University, New Delhi, in fulfillment of the requirement for the award of the degree of Doctor of Philosophy, embodies faithful record of original research work carried out by **Shuchi Midha.** She has worked under my guidance and supervision. This work is original and has not been submitted so far in part or full for any other degree or diploma of any other university.

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LIST OF PUBLICATIONS

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5) Subhash Chandra, Manpreet Kaur, **Shuchi Midha**, Rakesh Bhatnagar, Nirupama Banerjee-Bhatnagar, Evaluation of the ability of N-terminal fragment of lethal factor of *Bacillus anthracis* for delivery of Mycobacterium T cell antigen ESAT-6 into cytosol of antigen presenting cells to elicit effective cytotoxic T lymphocyte response, 1: ***Biochemical Biophysical Research Communications.* 2006** Dec 22; 351(3):702-7.

6) **Shuchi Midha,** Rajeev Mishra, Mohd. Azhar Aziz, Meenakshi Sharma, Ashish Mishra, Puneet Khandelwal, Rakesh Bhatnagar, Cloning, expression, and characterization of recombinant nitric oxide synthase-like protein from *Bacillus anthracis*, ***Biochemical and Biophysical Research Communications,* 2005**; 336 : 346–356.

Dedicated to my Family who taught me the first lesson of science that:

"The greatest discovery is you can alter your life by altering your attitude of mind....."

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Life has blessed me with these special people. This thesis is dedicated to them from a student still anxious to learn more.....

ABBREVIATIONS

ASC	Antibody Secreting Cell
Ab	Antibody
APS	Ammonium persulfate
APC	Antigen presenting cell
BSA	Bovine serum albumin
$^{\circ}C$	Degree Celsius
DAB	Diamino benzidine
DC	Dendritic cell
ddH_20	Double distilled water
DNA / RNA	Deoxyribose / ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
EDTA	Ethylene diamine tetraacetic acid
EF	Edema factor
ELISA	Enzyme linked immunosorbent assay
FCS	Fetal calf serum
HRP	Horseraddish peroxidase
HEPES	N-2-hydroxyethylpiperzine-N-2 ethane sulfonic acid
hr. / hrs.	Hour / Hours
IPTG	Isopropyl-β-D-thio-galactopyranoside
i.m	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous
kb	Kilo base
kDa	Kilo Dalton
LAMP1	Lysosome Associated Membrane Protein 1
LB	Luria Bertani medium
LF	Lethal factor
LT / Letx	Lethal toxin
M	Molarity
mCi	Milli Curie
mg	Milligram

MHC	Major Histocompatibility Complex
mM	Milli molar
MΦ	Macrophage
MTT	3-(4,5-dimethylthiazol-2-yl),-5-diphenyltetrazolium bromide
N	Normality
ng	Nanogram
Ni-NTA	Nickel - nitrilotriacetic acid
NKT	Natural Killer T-cell
O/N	Over night
PA	Protective antigen
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PMSF	Phenyl methylsulphonyl fluoride
rpm	Revolutions per minute
RNase	Ribonuclease
RPMI	Roosevelt Park Memorial Institute
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis
SFU	Spot Forming Unit
TAE	Tris-acetate EDTA
TE	Tris EDTA
TEMED	N, N, N', N' tetramethyl ethylene diamine
TPA	Tissue Plasminogen Activator
TNA	Toxin Neutralising Antibody.
x *g*	Times gravity (Centrifugal force)
β-ME	Beta mercaptoethanol
µg	Microgram
µl	Microlitre
µM	Micromolar
%	Percentage
~	Approximately

CONTENTS:

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

During the past decade, emphasis on the study of pathogenesis of infectious diseases has shifted focus to determine the mechanism of action of microbial virulence factors that helps reveal their *modus operandi* and highlights important features of the affected host target. These virulence factors that are 'shaped up', by evolution, around a particular target molecule, play a critical role in regulating pathogen stability, replication and persistence. These factors are particularly insightful since they add temporal and spatial elements to host-pathogen interactions. They integrate the activities of the target cells by binding to high-affinity receptors whose direct consequence is microbial perturbation of host defense function i.e. immune evasion or immune suppression.

This is also the case for *Bacillus anthracis*, which causes toxin-mediated immuno-modulation of host immune response, necrosis or apoptosis of various host cell types and, capsule-mediated inhibition of phagocytosis by antigen presenting cells (Young *et al.* 2007). Anthrax has a special place in the history of medicine and biology because its study has resulted in the foundation of medical microbiology and immunology (Kauffmann *et al.* 2005). The recent revival of the study of anthrax pathogenesis, caused by the bioterrorist attack in 2001 in US (Matsumoto *et al.* 2003), has stimulated a great advance in our understanding of the microorganism-mammalian immune-defense interface, to the extent that anthrax can be considered a paradigm of the pathogenesis of other infectious diseases.

Anthrax is considered to be among the most likely threat agents of bioterrorism and, as such, has been designated as a category A agent, along with the agents of smallpox, botulism, plague, tularemia, and certain viral hemorrhagic fevers, by the Centers for Disease Control and Prevention (CDC) (MMWR report). The threat was realized in the United States in 2001 when attacks using aerosolized anthrax spores resulted in 11 cases of the inhalational form of disease; 5 of the 11 patients died (Jernigan *et al.*2001). Although multiple antimicrobials possess activity against *Bacillus anthracis,* they do not provide complete protection against disease, because systemic illness results from the effects of toxins produced by the organism during infection. Patients with inhalational anthrax appear to reach a critical

threshold in the pathogenic process beyond which survival is unlikely (Jernigan *et al.*2001; Inglesby *et al.* 2002). Therefore, an early, multifaceted approach to therapy may be necessary to optimize outcomes in these cases. One such approach is to target key steps in the process of intoxication.

Cellular intoxication occurs as result of the action of two anthrax toxins, edema toxin (Etx) and lethal toxin (Letx), derived from the combination of three different factors: protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa) and lethal factor (LF, 90 kDa). PA binds to the cell-surface receptors, tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, which are expressed as different isoforms by many cell types, including immune cells (Collier *et al.* 2003; Bradley *et al.*2001; Liu *et al.* 2003; Scobie *et al.* 2003). TEM8 and CMG2 interact with LDL-receptor related protein (LRP) 6, which is essential for EF and LF endocytosis (Wei *et al.* 2006).

PA consists of four domains, of which the 20 kDa C-terminal domain is released by the proteolytic cleavage of an exposed loop (Klimpel *et al.* 1992), resulting in the spontaneous oligomerization of truncated PA (PA63) into heptamers [$(PA63)_7$] that bind to EF and LF (Collier *et al.* 2003). The $(PA63)_7$–EF complexes, or the $(PA63)_7$–LF complexes, enter lipid rafts, inducing their endocytosis inside acidic compartments (Abrami *et al.* 2005). The low pH of the endosomal lumen triggers a conformational change in the complex, with the insertion of a flexible loop of each PA molecule into the lipid bilayer and the translocation of EF and LF into the cytosol (Collier *et al.* 2003; Abrami *et al.* 2005).

EF is a calmodulin-dependent adenylate cyclase (Leppla *et al.* 1982; Drum *et al.* 2002) that causes a prolonged increase of cytosolic cyclic adenosine monophosphate (cAMP), a strategy successfully evolved by several other bacteria to disrupt a wide array of physiological processes. LF is a metalloprotease which cleaves most isoforms of mitogen-activated protein kinase kinases (MAPKKs, also known as MEKs) close to their N-terminus (Vitale *et al.* 2000), but this does not exclude the possibility that it affects other cytosolic proteins. MEKs belong to a major signaling pathway linking the activation of membrane receptors to the transcription of several genes, including those encoding pro-inflammatory cytokines and other proteins involved in the immune response (Chang *et al.* 2001, Zhang *et al.* 2005). Mitogen-activated protein kinases (MAPKs) are the substrates of MEKs, to

which they bind through specific interactions involving the kinase domain and the same N-terminal tail recognized by LF (Montecucco *et al.* 2004). LF seems to interact with MEKs through the kinase domain in addition to the N-terminal tail (Montecucco *et al.* 2004). Thus, this mode of interaction seems to have been cleverly shaped by evolution in such a way that MEKs cannot escape LF recognition through mutations without severe consequences for cell physiology.

As a consequence of concerns over the severity of human infection caused by these toxins and the illicit use of this organism, considerable effort has focused on the development of therapies capable of conferring protection against anthrax. Antibiotics are available, but have to be administered at the earliest possible opportunity, often prior to the advent of symptoms, to be effective (Smith *et al.* 1954). The emergence of antibiotic-resistant strains has prompted researchers to pursue additional therapeutic options (Stepanov *et al.* 1996). The principal approach has been the development of peptides and antibodies, which inhibit the activity of the organisms' tripartite toxin system (Baillie *et al.* 2001a; Hull *et al.* 2005). Although these approaches are effective, they suffer from the same drawback as antibiotics, in that they need to be given repeatedly to maintain an adequate level of protection.

Vaccination is therefore, the most cost-effective form of mass protection. While Pasteur developed the first anthrax animal vaccine in 1881, human vaccines did not emerge until the middle of the twentieth century (Pasteur 1881; Baillie 2001b). The vaccines currently available provide effective protection, but suffer from problems of standardization; are relatively expensive to produce; and have been associated with transient side effects (Turnbull 2000; Baillie 2001b). In addition, the currently licensed US vaccine approved for use in humans against infection with *Bacillus anthracis* (Anthrax Vaccine Adsorbed-Biothrax) which is prepared by adsorbing filtered culture supernatants of the V770-NP1-R strain of *B. anthracis* to aluminum hydroxide (Alhydrogel), calls for a daunting series of three injections at short intervals followed by three injections at 6-month intervals. Although there is no direct evidence of the efficacy of this vaccine in humans, the low incidence of infection in vaccinated workers who are at risk of exposure to *B. anthracis* spores, such as laboratory workers, veterinarians, and those who work with hides, wool, and bone, suggests that this vaccine is efficacious (Friedlander *et*

al. 1999). In addition, the efficacy of AVA has been shown to vary in different animal models (Fellows *et al.* 2001).

On the other hand, the UK licensed vaccine suffers from the disadvantage of being incompletely characterized. It comprises trace amounts of LF and other bacterially derived, immunogenic antigens in addition to containing large amounts of PA, which have been shown to stimulate antibody responses in recipients, and might contribute to protection (Baillie *et al.* 2003, 2004). However, the presence of these additional proteins also accounts for the reactogenicity seen in some individuals (Turnbull 2000).

Protection against anthrax has been associated with the development of anti-PA antibodies (Welkos *et al.* 2001). Also, recent work has demonstrated that phagocytosis of spores and subsequent killing by murine macrophages is enhanced by anti-PA antibodies, which might recognize PA non-specifically associated with the exosporium (Welkos *et al.* 2002; Cote *et al.* 2005; Kang *et al.* 2005). The ability to kill the organism upon germination can thus prevent disease progression and toxemia. Therefore, the contribution of this functionality by a vaccine to the overall level of protection following immunization with PA needs to be determined. However, the identification correlates of protection are driven by the scarcity of human inhalational anthrax, which makes it impractical to perform human protection studies with these vaccines. For this reason, the Food and Drug Administration in the United States has decided that any new vaccine against anthrax can be licensed using protection data from two relevant animal models, supported by well-defined correlates of protection (Food and Drug Administration 2002).

However, in addition there are certain other performance requirements of biodefense vaccines that are somewhat different from those for conventional vaccines. Some key features of biodefense vaccines to be developed for civilian use include (*i*) the rapidity by which a protective immune response can be elicited; (*ii*) the degree to which the vaccine, when administered post-exposure, improves the clinical course of an exposed person; (*iii*) a very high benefit-to-risk ratio of vaccination in all segments of the population; (*iv*) the speed and ease of manufacture, distribution, and administration; and (*v*) the inherent stability of the formulated, final filled product to allow for long-term stockpiling.

For these reasons, a new form of vaccination, using DNA that contains the gene of interest, is under intensive investigation, because of its very high benefit-to-risk ratio as compared to the currently available vaccines. The asset of a DNA vaccine lies in the antigen processing and presentation, which is analogous to what occurs in a natural infection. Apart from this a DNA vaccine is a "clean" and "stable" vaccine with clearly defined components where there are no elaborate protein purification procedures (Mc Donnell *et al.* 1996; Wang *et al.* 1998; Mac Gregor *et al.* 1998; Boyer *et al.* 2000). Moreover, the demonstration that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models has engendered considerable excitement in the vaccine community.

Attempts have been made to design DNA vaccines against anthrax. However, the major strategies have been either limited to the usage of various adjuvants like, cation-lipids (Jimenez *et al.* 2007; Hermanson *et al.* 2004), CpG oligonucleotides (Klinman *et al.* 2003); or else focused on the DNA delivery by, gene-gun (Torres *et al.* 1999), liposomes (Sloat *et al.* 2006), poly-coglycoside particles (Ribeiro *et al.* 2007), with an aim to enhance the cellular as well as humoral immune responses. Systemic vaccination strategies based on prime boost regimen that imply a DNA prime followed by a booster with recombinant PA have been tested (Galloway *et al.* 2004; Hahn *et al.* 2004). Additionally, DNA vaccine encoding a fragment of LF has been shown to provide protection against lethal toxin challenge (Price *et al.* 2001). Although these vaccine regimes were tested for their ability to induce measurable antibody responses, the generation of effective cellular adaptive immunity arising as a result of DNA vaccination was not investigated.

Given these considerations we report here the application of antigen trafficking to various compartments of the cell with an ultimate goal of improving the humoral as well as cellular immune responses. There is an increasing body of evidence to suggest that both $CD8^+$ and $CD4^+$ T cells are critical for the generation of an effective immune response against an intracellular pathogen. Although both recognize non-native forms of the antigen in association with major histocompatibility complex (MHC) molecules, the presentation of the antigen to these two lymphocytic populations occurs through distinct pathways (Catherine *et al.* 2002). Processing of endogenous antigens can occur potentially through

proteasomal degradation and TAP transport to ER followed by binding to MHC I (Rock *et al.* 1994) whereas degradation and loading of the exogenous antigen within the endosomes leading to MHC II loading (Nutchern *et al.* 1990; Harding *et al.* 1993).

Therefore, based on the hypothesis that immune outcome can be influenced by the form of antigen administered and its ability to access various antigen-processing pathways, we mediated the targeting of 63kDa fragment of protective antigen (PA) of *Bacillus anthracis* to various subcellular locations by DNA chimeras bearing a set of signal sequences. These targeting signals namely, LAMP1 (Lysosome Associated Membrane Protein 1), TPA (Tissue Plasminogen activator) and Ubiquitin encoded various forms of PA viz. lysosomal, secreted, and cytosolic, respectively.

Table 1. DNA constructs and the targeting signals attached.

PLASMID NAME	EXPRESSED PRODUCT
pTPA-PA63	N-terminal TPA signal, and 63 kDa mature protein.
pPA63-Native	63 kDa mature protein.
pLAMP1-PA63	C-terminal LAMP1 membrane anchor and 63 kDa mature protein.
pUQ-PA63	N-terminal Ubiquitin leader and 63 kDa mature protein.
pTPA-PA63-LAMP1	N-terminal TPA signal, C-terminal LAMP1membrane anchor and 63kDa mature protein.

Our further studies hinged on the observations that ATR resides in the glycerolipidic i.e. non-raft regions of the plasma membrane. However, upon binding and heptamerization of PA, the toxin-receptor complex associates with lipid-raft like domains and undergoes rapid endocytosis via clathrin-dependent pathway (Abrami *et al.* 2003). This process subsequently leads to the translocation of the enzymatic subunits into the cytosol through the protective antigen pore. This kind of behavior parallels B-cell receptor which also undergoes ligand-dependent clustering and raft association (Cheng *et al.* 1999), and is subsequently internalized via clathrin dependent mechanism (Stoddart *et al.* 2002).

The observation that ATR couples raft translocation and mediates endocytosis of EF/LF along with the oligomerization of PA made us hypothesize that a DNA chimera encoding protease-cleaved fragment of PA (PA63) attached to a C-terminal glycosylphosphatidyl inositol (GPI) anchor sequence might ensure lateral association of the cell-surface GPI-anchored PA with liquid-ordered, cholesterol- and sphingolipid-rich domains or rafts. Biochemical, morphological and functional approaches have been able to trace that GPI-anchored protein rich rafts are transported down to the endocytic pathway to reducing late endosomes in mammalian cells (Fivaz *et al.* 2002). Apart from that, GPI-anchoring has been attributed to be a positive signal for internalization into rab5-independent (rab5, a small GTPase located in early endosomes) tubular-vesicular endosomes also responsible for fluid-phase uptake (Sabharanjak *et al.* 2002). Therefore, adoption of such an approach opens up a possibility that GPI anchored PA will undergo its natural cycle upon endocytosis that might improve DNA vaccine potency. As a matter of fact, it is also well documented that CD1 molecules (MHC-I like glycoproteins) survey the endocytic pathway to intersect and bind lipid antigens (Barral *et al.* 2007). Both human and muroid CD1d molecules (Group 2 CD1 molecules) have been shown to bind to GPI-anchored proteins and present them to a variety of NKT cells that function against infection with pathogens (Schofield *et al.* 1999). Group 2 CD1 (CD1d) molecules have also been shown to control humoral immunity against parasites (Hansen *et al.* 2003), elicit cell-mediated immune responses against tumors (Cui *et al.* 1997), and induce secretion of large amounts of both inflammatory IFN-γ and immunoregulatory interleukin (IL)-4 and IL-10 cytokines (Yoshimoto *et al.* 1994; Wilson *et al.* 1998). Thus, GPI-anchoring of PA might as well aid its presentation in context with CD1 molecules that have the advantage of limited allelic polymorphism yet specialized to bind and present a large repertoire of lipids and glycolipids to T cells.

Based on this rationale we constructed DNA chimera encoding GPI-anchored form of PA utilizing the mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. The GPI signal appears to have been conserved, however, a mammalian GPI signal was specifically attached keeping in mind earlier studies that demonstrate that GPI signals from parasitic protozoa are not recognized by mammalian cells (Moran *et al.* 1994). We also designed a chimera in which PA

was attached with an N-terminal TPA leader and C-terminal GPI anchor. TPA is expressed by vascular smooth muscle and binds to a specific cellular receptor p63, which occurs in fibroblasts as an intracellular protein associated with the ER (Schweizer *et al.* 1995; Razzaq *et al.* 2003). The TPA leader peptide can therefore, target the expressed antigen directly to the ER thus, obviating the need for the antigen to be processed and translocated to this structure (Ciernik *et al.* 1996). So, we wanted to find out what impact, if any, it would have on DNA vaccine potency as ER is also the site where new CD1d molecules (the only muroid CD1 molecule) are synthesized.

Therefore, with these observations in mind we designed the present study and we successfully illustrated the generation of not only classical MHC II-restricted immunoglobulin (Ig) responses but also non-classical CD1d-restricted Ig responses. A significant correlation was found between survival percentage and post-challenge anti-PA titers and TNA titers. Overall, immune kinetics pointed that differential processing through various compartments gave rise to qualitative differences in the immune response generated by various chimeras.

2. AIMS & OBJECTIVES

The main objectives of the work are as follows:

1) Cloning of the PA63 gene fragment (protease-cleaved fragment of protective antigen) in DNA plasmids bearing various signal sequences for targeting the expressed protein antigen to various sub-cellular locations.
2) Evaluation of the authenticity of the DNA constructs by transient transfection in J774A.1 mouse MΦ-like cells followed by subcellular fractionation and immunoblot analysis.
3) Characterization of the cellular and humoral immune responses generated by the DNA constructs in mouse model.
4) Evaluation of the ability of the DNA chimeras to confer protection against lethal toxin challenge.
5) Prediction of the variables of the correlates of protective immunity.

CHAPTER 2

REVIEW OF LITERATURE

1. A BRIEF HISTORY OF ANTHRAX

Anthrax is a zoonotic disease caused by *Bacillus anthracis,* a large gram-positive bacillus that in its spore form can persist in nature for prolonged period, possibly years. Humans, mammals, several birds, reptiles and amphibian species are susceptible to infection to varying degrees. Domestic livestock and wild herbivores, for example, elephants and hippopotamus, are especially vulnerable (Turnbull 1990). Human cases arise due to contact with the infected animals or animal products (that is, wool, hide, hair, bone and skin), by consuming undercooked meat or by inhalation of air-borne spores. Morbidity is extremely low in cutaneous cases, less than 5% if antibiotics are given. However, inhalation anthrax is almost 100% fatal, and gastrointestinal cases have mortality rate of from 25% to 75%.

Although anthrax has been known since antiquity, it was not always clearly distinguished from other diseases with similar manifestations. Scholars have characterized the fifth and sixth biblical plagues as well as the "burning plague" described in Homer's *Iliad* as anthrax. However, it was Virgil (70-19 BC) who provided one of the earliest and most detailed descriptions of an anthrax epidemic in his *Georgics*. Virgil also noted that the disease could spread to humans.

Over the next fifteen hundred years, Europe witnessed sporadic outbreaks of anthrax, with the most acute outbreaks occurring in fourteenth-century Germany and seventeenth-century Russia and central Europe. Despite the threat these outbreaks posed to livestock, it was only in 1769 that Jean Fournier classified the disease as anthrax or *charbon malin*, a name undoubtedly derived from the black lesions characteristic of cutaneous anthrax. Fournier also noted a link between those who worked with raw animal hair or wool and susceptibility to anthrax.

Anthrax continued to affect livestock and humans throughout the Middle Ages. During the 18th century, anthrax epidemics destroyed approximately half the sheep population in Europe (Dirckx 1981). In Victorian England the disease came to be known as "woolsorters' disease" because it was frequently observed among mill workers exposed to animal fibers contaminated with *B. anthracis* spores. However, the name is somewhat a misnomer: the infection was more often a result of contact with goat hair or alpaca than with wool and sheep (Brachman 1980). Other names for the disease included "ragpickers' disease," "milzbrand", "black bain", "tanners' disease," and "Siberian (splenic) fever".

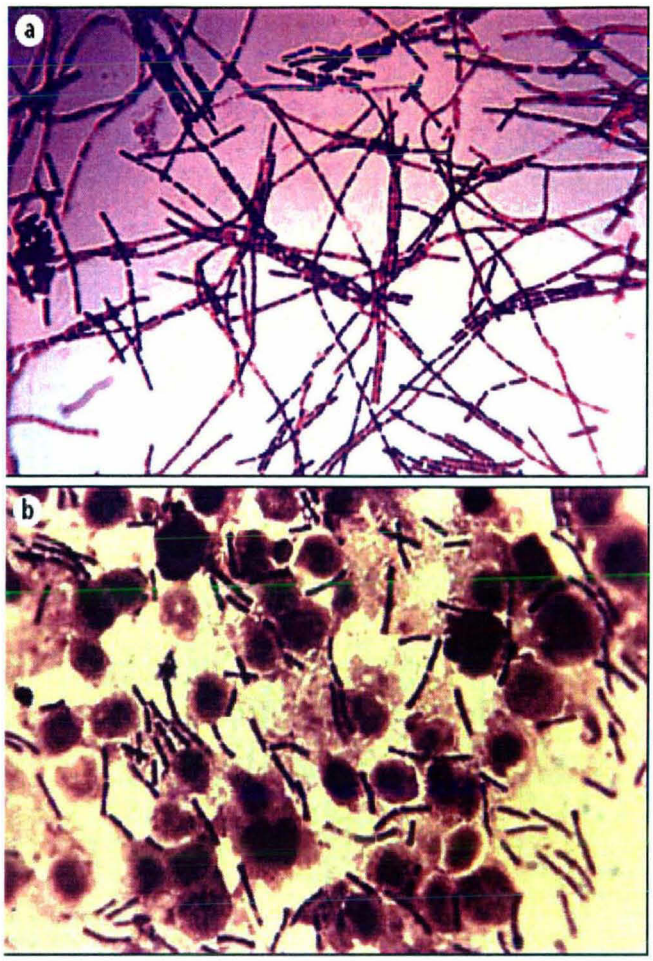
In the 19th century, anthrax was a major point of interest in developing biomedical research. In 1850, Pierre Rayer and Casimir-Joseph Davaine discovered small filiform bodies "about twice the length of a blood corpuscle" in the circulatory system of sheep afflicted with anthrax (Carter 1988). Initially, this discovery was not given any significance, as the filiform bodies were regarded as disease products. However, Davaine subsequently suggested that the corpuscles described were organisms causing the disease.

By the mid 1870s, most researchers believed that anthrax was an infectious disease but there was still disagreement as to its specific cause. In 1876, Robert Koch, a Prussian physician, isolated the anthrax bacillus and pointed out that the bacillus could form spores which remained viable, even in hostile environments. He used suspended drop culture techniques and was able to trace the complete life cycle of the anthrax bacillus for the first time in history (Carter 1988). Furthermore, he postulated that the anthrax bacillus could be transmitted from one host to another, and in 1877 he grew the organism in vitro and induced the disease in healthy animals by inoculating them with material from these bacterial cultures. Anthrax was the first disease for which etiological agent was determined and based on these findings Koch gave his famous postulates.

William Greenfield was the first to immunize livestock successfully against anthrax in 1880. However, credit for the use of a live vaccine against anthrax is usually given to Louis Pasteur who tested a heat-cured vaccine on sheep in 1881. By the late twentieth century, extensive animal vaccination programs led to an overall decline in anthrax although the disease still occurred in poor and unstable regions (between 1978 and 1980, for example, a civil war in Zimbabwe caused a breakdown in veterinary care which then resulted in an anthrax epidemic which spread from animals to humans).

Metchnikoff (1905), using transparent tissues of live animals attached to his microscopic stage, employed the anthrax bacilli to show that his newly discovered large blood cells exited the circulatory system (diapedesis), migrated towards the bacilli (chemotaxis) and ingested the virulent organisms (phagocytosis). He called these unique cells macrophages. In 1939, Sterne reported his development of an animal vaccine that is a spore suspension of an avirulent, non-encapsulated live strain. This is the animal vaccine currently recommended for use in animals.

2. MICROBIOLOGY



The *Bacillus* genus has 70 species, including *B. cereus*, *B. subtilis*, *B. anthracis*, and *B. thuringiensis*. Some species have been lost to other genera by proposals for new *Bacillus* species. Members of the *B. cereus* group-*B. anthracis*, *B. cereus*, and *B. thuringiensis*- are really pathovars of a single species (Logan *et al.* 2003). However, it is convenient to classify *B. anthracis* within the *B. cereus* group, which then comprises *B. cereus*, *B. anthracis*, *B. thuringiensis*, and *B. mycoides* by phenotype (Turnbull 1999).

Fig.1. Gram stain of B.anthracis from culture (a) from culture, (b) from infected tissue

B. anthracis is a large, aerobic, gram-positive, spore-forming, non-motile bacillus. The non-flagellated vegetative bacillus is 1–1.5 by 3–10 μm in size and is the only obligate pathogen within the genus (Baillie & Read 2001). In infected blood or tissues, the bacilli are frequently present in short chains, surrounded by the polypeptide capsule, which can be visualized under the microscope if stained with polychrome methylene blue (McFadyean stain; *Figure 2)* or highlighted with India ink. However, in stained smears from anthrax colonies grown on plates, no capsule is identified unless the medium contains 0.7% bicarbonate or 5% serum and the plates are incubated in a 5% to 10% carbon dioxide atmosphere (Carman *et al.* 1985).

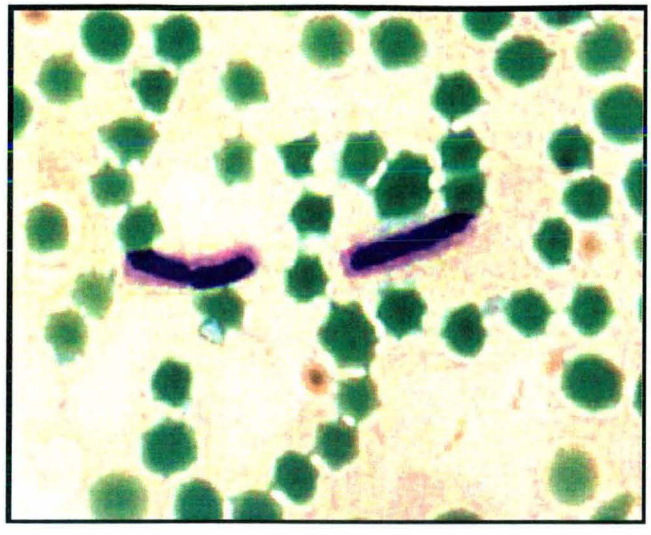


Figure2. McFadyean capsule stain of Bacillus anthracis, grown at 35°C in defibrinated horse blood. Courtesy of CDC/Larry Stauffer/Public Health Image Library.

It is impossible to discriminate between species of the *B. cereus* group by 16S rRNA sequencing, although *B. anthracis* can be distinguished by multiple-locus variable-number tandem repeat analysis and amplified fragment-length polymorphism (Spencer 2003; Keim *et al.* 2000). *B. anthracis* is one of the most molecularly monomorphic bacteria known. However, based on variable numbers of tandem repeats in the region of the *VrrA* gene, all known *B. anthracis* strains can be separated into five categories for geographical identification (Jackson *et al.* 1998).

Unlike other members of the *B. cereus* group, *B. anthracis* is non-motile and non-hemolytic on sheep blood agar. It grows readily on a variety of laboratory media at 37°C, forming typical white-gray colonies with an oval, slightly granular appearance. The colonies of *B. anthracis* are about 2 mm in diameter and somewhat smaller when compared with those of other species in the *B. cereus* group *(Figure 3B).* It is generally easy to distinguish the virulent *B. anthracis* from members of the *B. cereus* group by colony morphology, susceptibility to the diagnostic gamma phage *(Figure 3A),* and the ability to produce the characteristic capsule as visualized by the McFadyean stain.

Bacilli form spores when local nutrients in the environment are exhausted or when anthrax-infected body fluids are exposed to ambient air (Spencer 2003). However, under favorable conditions, anthrax spores germinate and rapidly multiply into vegetative anthrax bacilli. Vegetative bacilli have poor survival outside an animal or human host; colony counts decline rapidly to undetectable numbers within 24 hours after inoculation with water. This clearly contrasts with the

environmentally hardy properties of the *B. anthracis* spore, which can survive for decades (Inglesby *et al.* 1999; Titball *et al.* 1991; Logan *et al.* 2003).

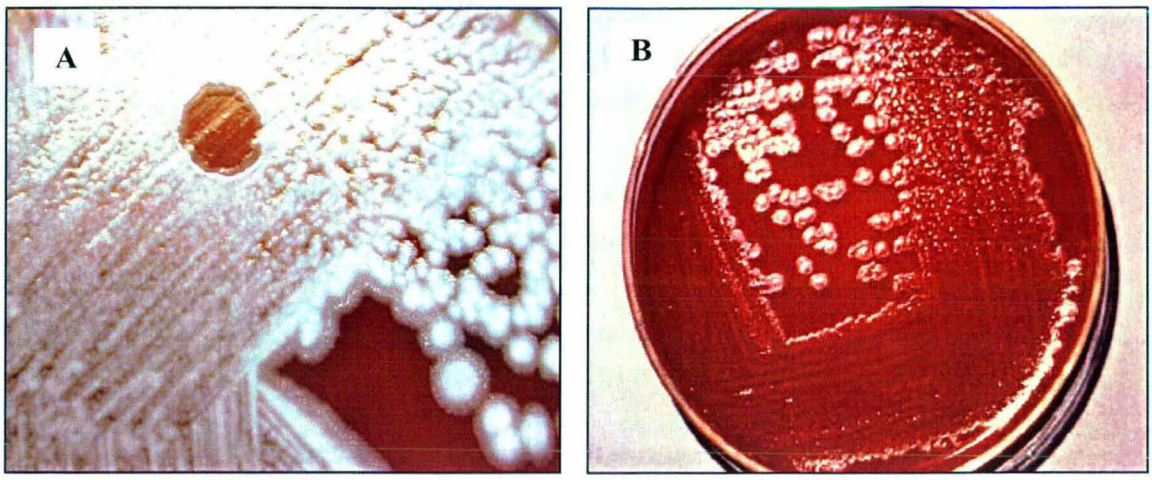


Figure 3. (A) Gamma phage lysis of Bacillus anthracis on sheep blood agar. (B). Colonies of Bacillus anthracis on sheep blood agar plate. Courtesy of CDC/Public Health Image Library

3. CLINICAL ASPECTS OF THE DISEASE

There are primarily three forms of anthrax disease- cutaneous, gastrointestinal and inhalational. However, another clinical manifestation of the disease is meningitis.

3.1. Cutaneous anthrax. Typically, cutaneous anthrax occurs on the exposed parts of the body. Although a single lesion is typical, some patients may develop multiple discrete lesions. The lesion begins innocuously with a small papule that the patient may first notice because of puritus. The papule develops after several days into a small vesicle or occasionally into several vesicles that then coalesce to form a ring. The area may be surrounded by a small ring of erythema and possibly some edema. The pruritus may continue but there is no pain unless there is secondary infection or a significant degree of local edema is present. A small dark area can be seen beneath the center of the vesicle. Eventually the vesicle or the vesicular ring ruptures, discharging a clear fluid and revealing a depressed black necrotic central area known as eschar *(Figure 4)*. After 1-2 weeks, the lesion dries and the eschar begins to loosen and shortly thereafter separates revealing a permanent scar.

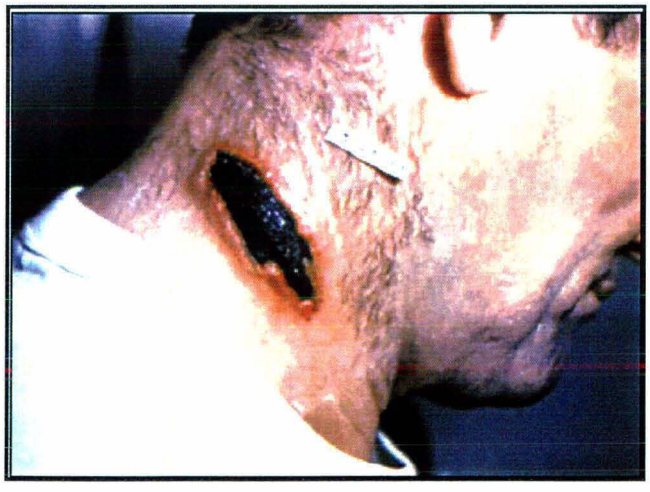


Figure 4. The eschar of cutaneous anthrax. Courtesy of CDC/Public Health Image Library.

3.2. Inhalational anthrax. This form shows a biphasic clinical pattern with a benign initial phase followed by an acute second phase that is almost always fatal. The initial phase begins as a non-specific illness consisting of malaise, fatigue, myalgia, mild fever, non-productive cough and occasionally a sensation of precordial oppression. This illness may resemble as the mild upper respiratory track

infection such as cold or the flu. After 2-4 days the patient may show signs of improvement. However, then there is a sudden onset of severe respiratory distress with dyspnea, cyanosis, respiratory stridor and profound diaphoresis. In several cases subcutaneous edema of the chest and neck has been described. The pulse, respiratory rate and temperature become elevated. Physical examination reveals moist crepitant rales over the lungs and possibly evidence of plural effusions *(Figure 5)*. Death occurs in most patients with inhalation anthrax within 24 hours after the onset of the acute phase.

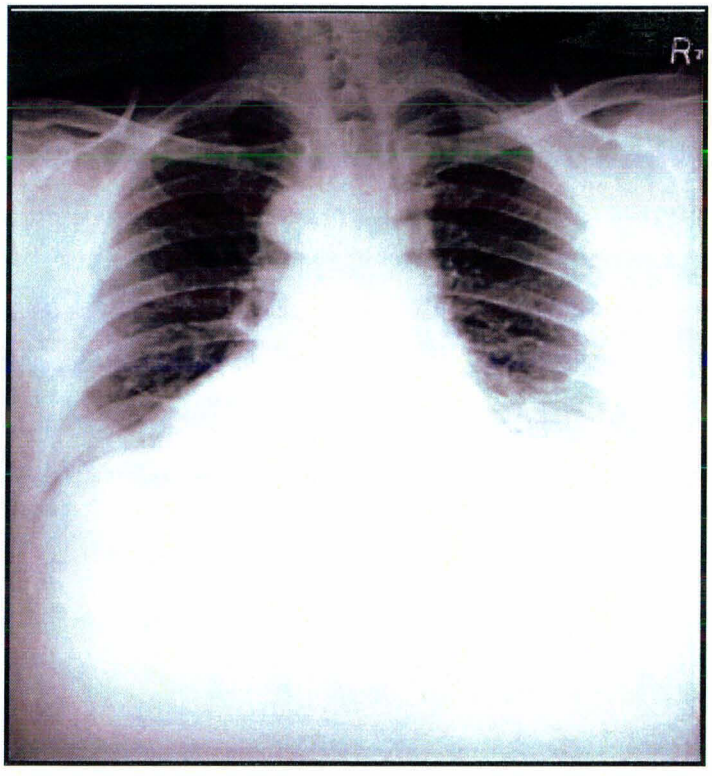


Figure5: A postero-anterior chest radiograph taken shortly after onset of inhalational anthrax in a 46-year-old man who contracted the disease from working in a goat hair processing mill. The radiograph reveals bilateral pulmonary effusion and a widened mediastinum, which are hallmarks of the disease process. Courtesy of CDC/Arthur E. Kaye/Public Health Image Library.

3.3. Gastrointestinal anthrax. There are two clinical presentations of the disease resulting from the ingestion of *B. anthracis*: abdominal and oropharyngeal. The symptoms of abdominal anthrax are initially non-specific and include nausea, vomiting, anorexia and fever. With the progression of the disease, abdominal pain, hematemesis and bloody diarrhea develops. Ascites may be present. Occasionally, the symptoms and the signs resemble an acute surgical abdomen. With further progression, toxemia develops with shock, cyanosis and death. The time from onset of symptoms to death has frequently varied from 2 to 5 days. In oropharyngeal

anthrax patients experience fever, submandibular edema, cervical lymphadenopathy and anorexia. Some reports describe the presence of acute inflammatory lesions in the oral cavity and or oropharynx. The mortality rate for gastrointestinal anthrax has been reported to vary from 25 to 60%.

3.4. Anthrax Meningitis. The hemorrhagic meningitis is striking and is often referred to as the "cardinal's cap" *(Figure 6)* (Abramova *et al.* 1993). When tissue from patients with disseminated anthrax is evaluated, light microscopy finds hemorrhage, edema, necrosis, fibrin deposition, and a variable degree of inflammatory cell infiltrate, predominantly consisting of neutrophils. Splenic lesions show fibrinopurulent necrosis and depletion of white pulp. The hemorrhagic meningitis is characterized by hemorrhage, fibrinopurulent inflammation, and a myriad of bacilli in and around blood vessels (Abramova *et al.* 1993). Necrotizing vasculitis and disseminated intravascular coagulation may also occur. Touch preparations from tissue and blood smears also demonstrate a myriad of typically "box car"–shaped, encapsulated gram-positive bacilli.

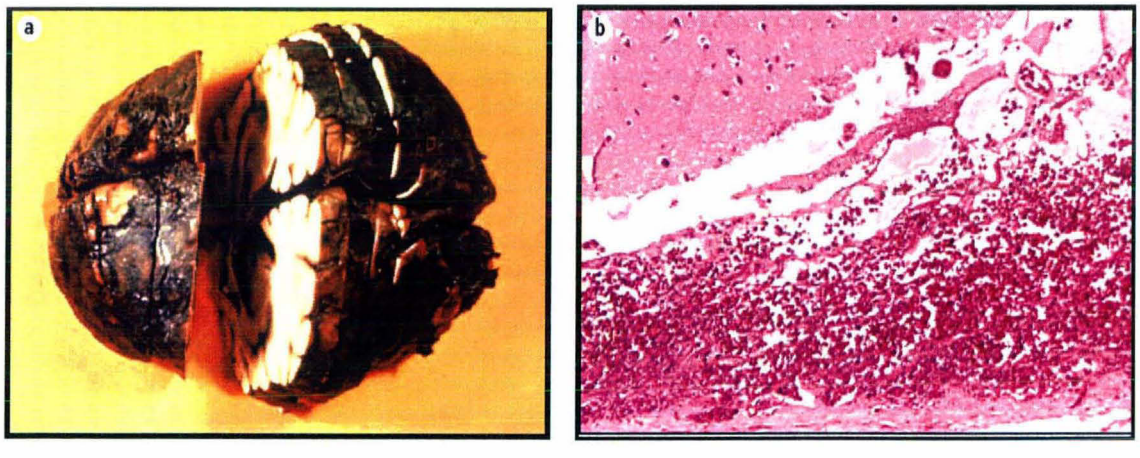


Figure6: Hemorrhagic meningitis due to inhalational anthrax. (a) Gross pathology of a formalin-fixed and cut brain. Courtesy of CDC/Public Health Image Library. (b) photomicrograph of meninges, ×125. Courtesy of CDC/Dr. LaForce/Public Health mage Library.

4. SUSCEPTIBILITY OF ANIMALS TO ANTHRAX INFECTION

Anthrax is primarily a disease of animals. Cattle are most susceptible to its infection. Horse and swine are susceptible but are less commonly affected. Wild deer and other gregarious herbivores are liable to occasional outbreaks. Rabbits, guinea pigs and white mice are susceptible in that order and are fatally affected by the subcutaneous inoculation of a very small number of virulent bacilli. Carnivores, though possessing greater resistance than herbivores are nonetheless susceptible. Interestingly, certain animals possess a marked natural resistance to anthrax. Most rats are quite resistant especially white rat. Dog is only slightly susceptible. Birds especially pigeons are not easily affected. Frogs are completely resistant but toads are very susceptible.

Animals usually acquire anthrax by the ingestion of contaminated substances (feed, grass, water and infected carcasses) (Turnbull 1996). They suffer most commonly from the gastrointestinal form of the disease. Cutaneous anthrax can occur, however, through the bite of bloodsucking flies. In highly susceptible animals, the disease frequently runs a hyperacute course, and signs of illness may be absent until shortly (sometimes in as little as one hour), before death. The animal then appears toxemic and occasionally after mild seizures becomes comatose and dies. In the more resistant species such as the pigs and the dogs, infection acquired by ingestion tends to become localized in the pharyngeal lymph nodes. The resulting edematous swelling may lead to death by occlusion of the airway. Alternatively, the disease may progress to fatal bacterimia and toxemia; or the animal may recover.

When acquired via the skin, through wounds or from fly bites, the disease usually progresses to death before eschers, carbuncles or other localized manifestations may occur. In unimmunised guinea pigs, death usually occurs in 36-48 hours but partial immunity conferred by inadequate vaccination prolongs survival and leads to development of eschars after 6 or more days.

5. TRANSMISSION IN ANIMALS

Spores in soil or water cause most cases of anthrax. The usual cycle of infection consists of (1) the shedding of vegetative bacilli by an infected animal, (2)

sporulation, and (3) spread of infection to other animals by the spores, which may in the meantime have been dispersed by dust or the wind. Herbivores that play a central role in anthrax epizooties, generally acquire the disease by ingesting spores from the soil. As death approaches, hemorrhagic effusions from the nostrils, mouth and anus further contaminate the soil. Urine and milk occasionally contain the bacilli. Biting flies occasionally transmit anthrax mechanically from animal to animal. They finally result in transmission of spores from anthrax carcass to the leaves of shrubs and trees, which serve as food for other animal species.

Scavengers feeding on an infected carcass may deposit *B. anthracis* spores elsewhere. The unsporulated anthrax bacilli present in unopened carcasses die out rapidly as putrefaction proceeds, the precise rate depending on temperature (Christie 1987). When conditions are not conducive to immediate sporulation, the bacilli appear to die rapidly, even in aquatic environments (Turnbull 1990). It therefore seems unlikely that vegetative forms ingested by scavengers will survive passage through the intestinal tract, and limited experimental evidence supports this view (Anon 1978). Anthrax spores, however undoubtedly survive in the gut, and consequently scavengers play a major role in disseminating the organism. In regions of Africa in which enzootics occur, vultures in particular have been incriminated, and *B. anthracis* has been isolated on several occasions from the droppings of these birds known to have fed some hours earlier on anthrax carcasses (Turnbull 1990).

6. SUSCEPTIBILITY AND TRANSMISSION OF ANTHRAX IN HUMANS

Circumstantial evidence indicates that, in comparison with herbivores, man is moderately resistant to anthrax. The estimated human LD_{50} dose from inhaled small particle aerosol has been estimated to be 8,000 to 10,000 spores (Meselson *et al.* 1979). *B. anthracis* is primarily transported by contact or through airborne routes. In the pulmonary form of industrial anthrax, spores in dust clouds created from the handling of dry hides, skins, wool, bone meal and the like are inhaled by the workers. In cutaneous anthrax, it is generally believed that infection occurs via a small cut or abrasion. As a result, exposed regions of the body are most frequently affected and the site of infection often reflects the occupation of the patient. Workers

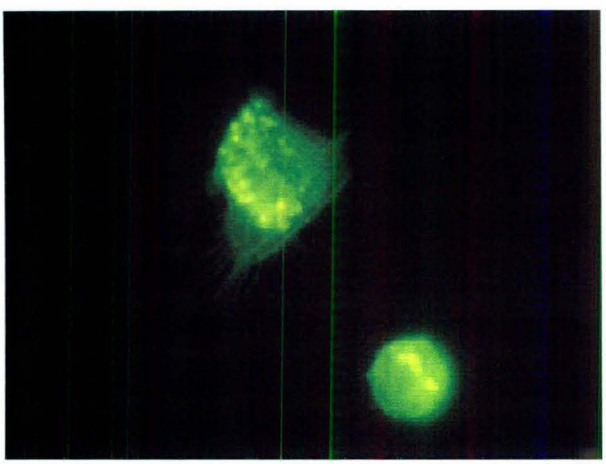
who carry hides or carcasses on their shoulders are prone to infection on the back of the neck. Handlers of other animal products tend to be infected on the arms or wrists, and occasionally the hands. Most lesions are found on the face, head and neck: less often affected are arms, and still less the trunk and lower limbs (Legge 1934; Davies 1983).

Insect vectors, such as horse flies, have been reported to transmit *B. anthracis* by means of mechanical transfer of the organism from contaminated foci to individuals or animals. The ability of stable flies and mosquitoes to transmit the disease has been confirmed in the laboratory (Sen *et al.* 1944; Turell *et al.* 1987). In some developing countries, the consumption of meat from animals that have died from anthrax, has resulted in the intestinal form of the disease (Davies 1982).

The most widely known recent 'outbreak' of human anthrax occurred in the United States in the fall of 2001. Prior to the bio-terrorist-related postal attacks, only 18 cases of inhalation anthrax were reported in the United States between 1900 and 1976 (Phipps *et al.* 2004). Altogether, there were 22 cases of laboratory-confirmed anthrax, half of which were inhalational in nature, with five cases proving fatal (45% case fatality ratio). Inhalational anthrax has an incubation period of 1–6 days, during which nonspecific symptoms of fever, sweat, fatigue, dyspenea, nonproductive cough and nausea have been reported (Abramova *et al.* 1993; Jernigan *et al.* 2002). These symptoms usually persist for 2 or 3 days, and in some cases, there is a short period of clinical improvement. This is followed by the sudden onset of increasing respiratory distress with dyspenea, stridor, cyanosis, increased chest pain and sweating. Respiratory distress is typically followed by the rapid onset of shock and death within 24–36 h. Mortality rates of 45–100% have historically been reported (Jernigan *et al.* 2002; Phipps *et al.* 2004).

7. PATHOGENESIS

Despite the early identification of *B. anthracis* as the cause of anthrax, its pathogenic process in humans is still ill-defined. The interaction between the spore and the macrophage is the key event in the disease process (Hanna 1997; Guidi-Rontani *et al.* 1999; Hanna *et al.* 1999).



Figur 7. Germination of B.anthracis inside alveolar macrophages. Infected macrophages were detected by staining F-actin with Oregon green-488 phalloidin (green). Germinated B. anthracis spores were stained with an anti-bacillus serum and a rhodamine-conjugated secondary antibody (red; co-localization of the two stains shows as yellow). [Adopted from Trends Microbiol 2002, 10:405-9]

Spores taken up by alveolar macrophages germinate in the phagolysosome (Guidi-Rontani *et al.* 2001) *(Figure.7)*. Recent data suggest that the spores, rather than being an inert structure, might actually interact with the innate immune system to promote germination and subsequent infection (Redmond *et al.* 2004; Baillie *et al.* 2005; Raines *et al.* 2006). Following germination, the organism elaborates a capsule and toxins, along with other products that enable it to escape the macrophage; suppress the host's immune system; and replicate and disseminate within the body to cause massive, terminal bacteraemia (Baillie & Read 2001) *(Figure. 8)*.

Image cytometry and expression technology using sensitive fluorescence-based reporter systems have demonstrated rapid onset of the expression of genes encoding virulence factors such as lethal factor (LF), protective antigen (PA), edema factor (EF) and the toxin *trans*-activator, AtxA (Guidi-Rontani *et al.* 1999). Moreover, *in vitro* experiments show that the survival of germinated spores and the loss of macrophage cell viability were associated with the expression of toxins (Guidi-Rontani *et al.* 2001). The lysis of macrophages harboring germinated spores occurs as a result of a cooperative toxin interaction involving edema toxin and lethal

toxin Thus the *B. anthracis* toxins presumably play a key role during early intracellular infection.

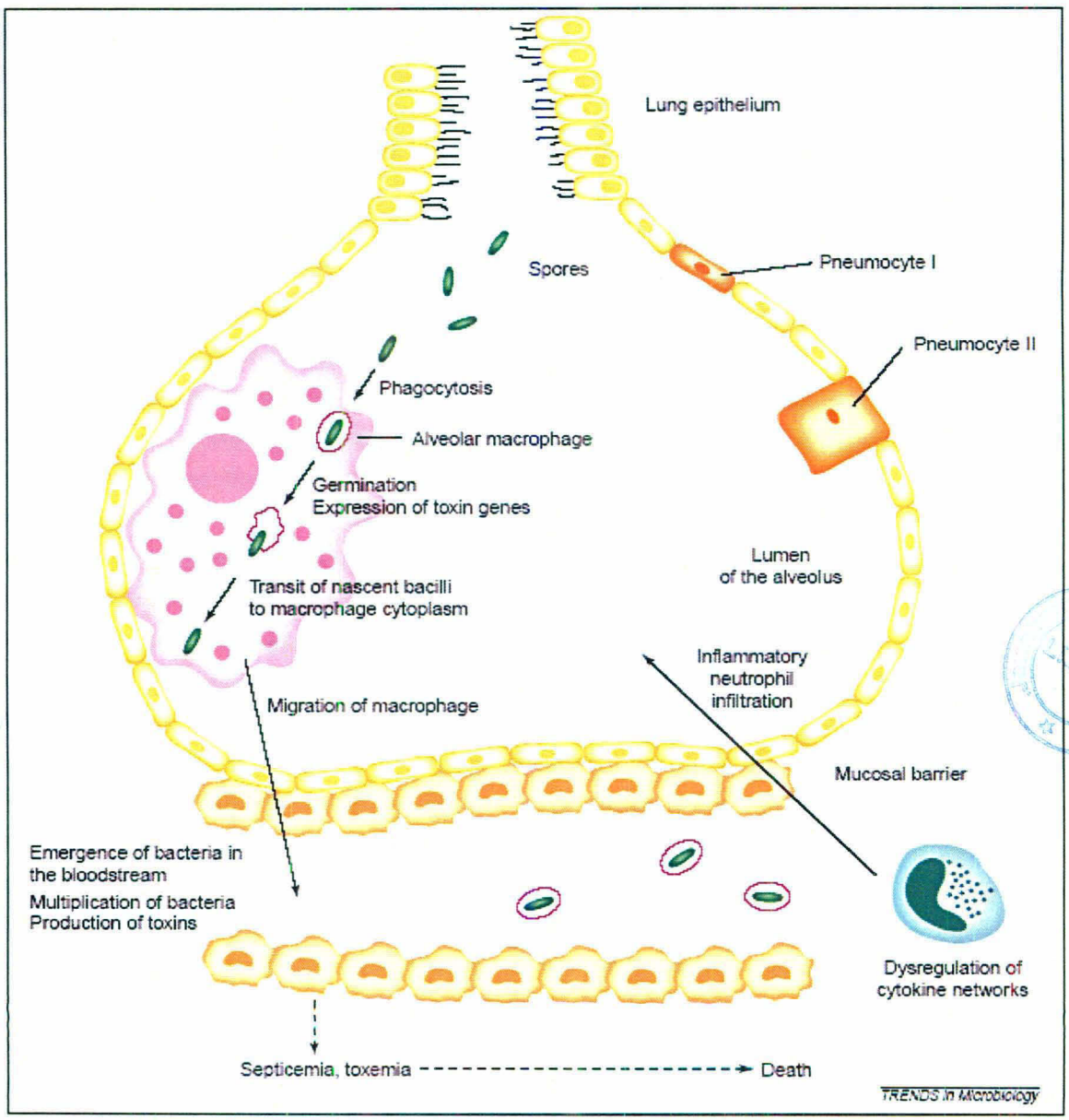


Figure 8. The molecular and cellular mechanisms involved in the early phase of Bacillus anthracis infection. B. anthracis spores are shown reaching the lumen of the alveolus. Once they reach the phagolysosome, after phagocytosis by alveolar macrophages, the spores germinate and produce edema toxin (Etx) and lethal toxin (Letx). Disruption of the phagolysosome membrane allows the transit of nascent bacilli into the macrophage cytoplasm. Bacteria that have escaped from the macrophage phagosome are carried from the primary site of infection by the migrating macrophage. Once the bacteria emerge in the bloodstream, they can multiply. As the infection progresses, bacteria spread through the blood and lymph causing the bacillemia and the toxemia characteristics of fatal anthrax infection. [Adopted from Trends Microbiol 2002, 10:405-9]

8. ANTHRAX TOXINS

The two anthrax toxins, edema toxin (Etx) and lethal toxin (Letx), derive from the combination of three different factors: protective antigen (PA, 83 kDa), edema factor (EF, 89 kDa) and lethal factor (LF, 90 kDa). PA binds to the cell-surface receptors tumor endothelium marker (TEM) 8 and capillary morphogenesis protein (CMG) 2, which are expressed as different isoforms by many cell types, including immune cells (Collier *et al.* 2003; Bradley *et al.*2001; Liu *et al.* 2003; Scobie *et al.* 2003). TEM8 and CMG2 interact with LDL-receptorrelated protein (LRP) 6, which is essential for EF and LF endocytosis (Wei *et al.* 2006) *(Figure 9)*

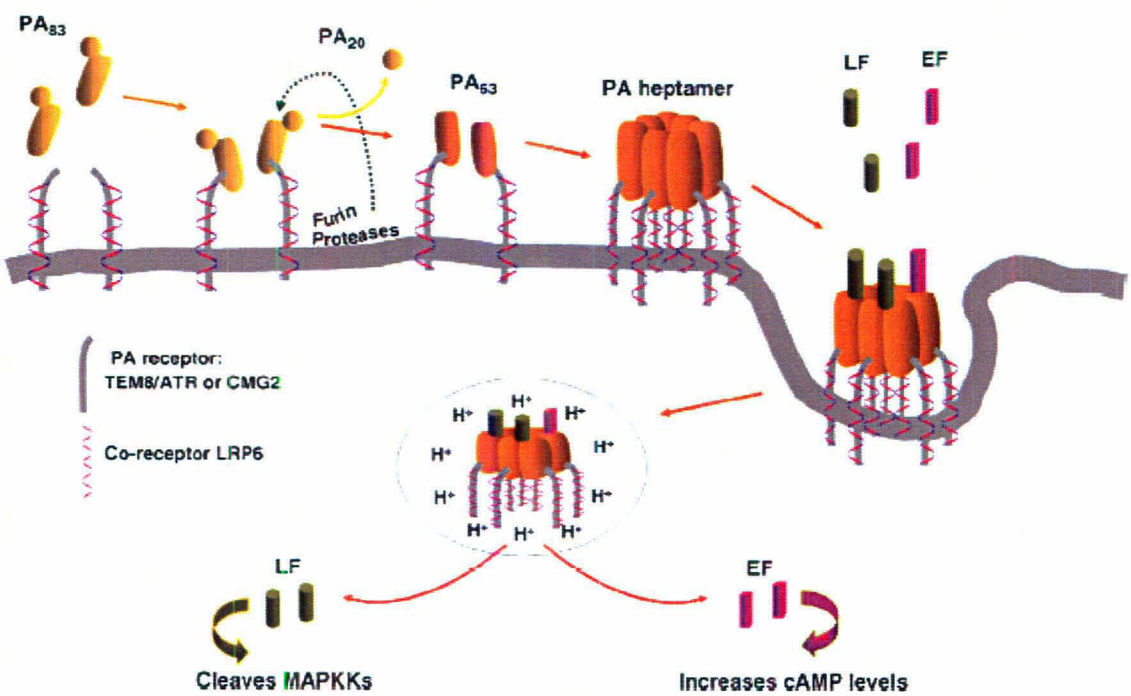


Figure 9. Model for the internalization of anthrax toxin. PA83 binds one of two cellular receptors, TEM8/ATR or CMG2. Upon binding, PA83 is cleaved by cellular furin proteases and the smaller fragment (PA20) is released. The larger fragment, PA63 forms a ring-shaped heptameric pre-pore, which can simultaneously bind up to three molecules of LF and/or EF. The toxin/receptor complex is then internalized and the endocytic vesicles are subsequently acidified, initiating a conformational change of the PA heptamer which converts it from the pre-pore into a mature pore that can allow entry of EF and/or LF toxin enzymes into the cytoplasm of target cells. LF is a protease targeting specific MAPKKs, whereas EF is an adenylate cyclase that catalyzes the formation cAMP. [Adopted from Int J Biochem & Cell Biol 2007, 39:20-24]

PA consists of four domains, of which the 20 kDa C-terminal domain is released by the proteolytic cleavage of an exposed loop (Klimpel *et al.* 1992), resulting in the spontaneous oligomerization of truncated PA (PA63) into heptamers [$(PA63)_7$] that bind to EF and LF (Collier *et al.* 2003). The $(PA63)_7$–EF complexes, or the $(PA63)_7$–LF complexes, enter lipid rafts, inducing their endocytosis inside acidic compartments (Abrami *et al.* 2005). The low pH of the endosomal lumen triggers a conformational change in the complex, with the insertion of a flexible loop of each PA molecule into the lipid bilayer and the translocation of EF and LF into the cytosol (Collier *et al.* 2003; Abrami *et al.* 2005).

EF is a calmodulin-dependent adenylate cyclase (Leppla *et al.* 1982; Drum *et al.* 2002) that causes a prolonged increase of cytosolic cyclic adenosine monophosphate (cAMP), a strategy successfully evolved by several other bacteria to disrupt a wide array of physiological processes. Upon calmodulin binding, EF undergoes structural rearrangements, unique among adenylate cyclases, which leads to its activation (Drum *et al.* 2000 & 2001; Ulmer *et al.* 2003). EF has a specific activity 1000-fold greater than mammalian adenylate cyclases and induces a substantial increase in conversion of intracellular ATP to cyclic AMP (cAMP). Subsequently, water homeostasis and cellular signalling pathways of the host are disrupted, leading to oedema during cutaneous anthrax infection (Leppla 1982 & 1984, Dixon *et al.* 1999). Additionally, Etx inhibits the ability of neutrophils to phagocytose bacilli and produce an oxidative burst (Wright *et al.* 1986; Cote *et al.* 2004) *(Figure. 10).* Several pathogenic bacteria encode virulence factors that are either adenylate cyclases or activate host adenylate cyclases (Ahuja *et al.* 2004). Despite this convergence on similar virulence mechanisms in distantly related bacterial pathogens, relatively little research has been devoted to Etx in the past. However, this is changing rapidly, and several groups have now reported on toxic effects of Etx.

LF is a metalloprotease which cleaves most isoforms of mitogen-activated protein kinase kinases (MAPKKs, also known as MEKs) close to their N-terminus (Vitale *et al.* 2000), but this does not exclude the possibility that it affects other cytosolic proteins. MEKs belong to a major signaling pathway linking the activation of membrane receptors to the transcription of several genes, including those encoding pro-inflammatory cytokines and other proteins involved in the immune

response (Chang *et al.* 2001, Zhang *et al.* 2005). Mitogen-activated protein kinases (MAPKs) are the substrates of MEKs, to which they bind through specific interactions involving the kinase domain and the same N-terminal tail recognized by LF (Montecucco *et al.* 2004). LF seems to interact with MEKs through the kinase domain in addition to the N-terminal tail (Montecucco *et al.* 2004). Thus, this mode of interaction seems to have been cleverly shaped by evolution in such a way that MEKs cannot escape LF recognition through mutations without severe consequences for cell physiology.

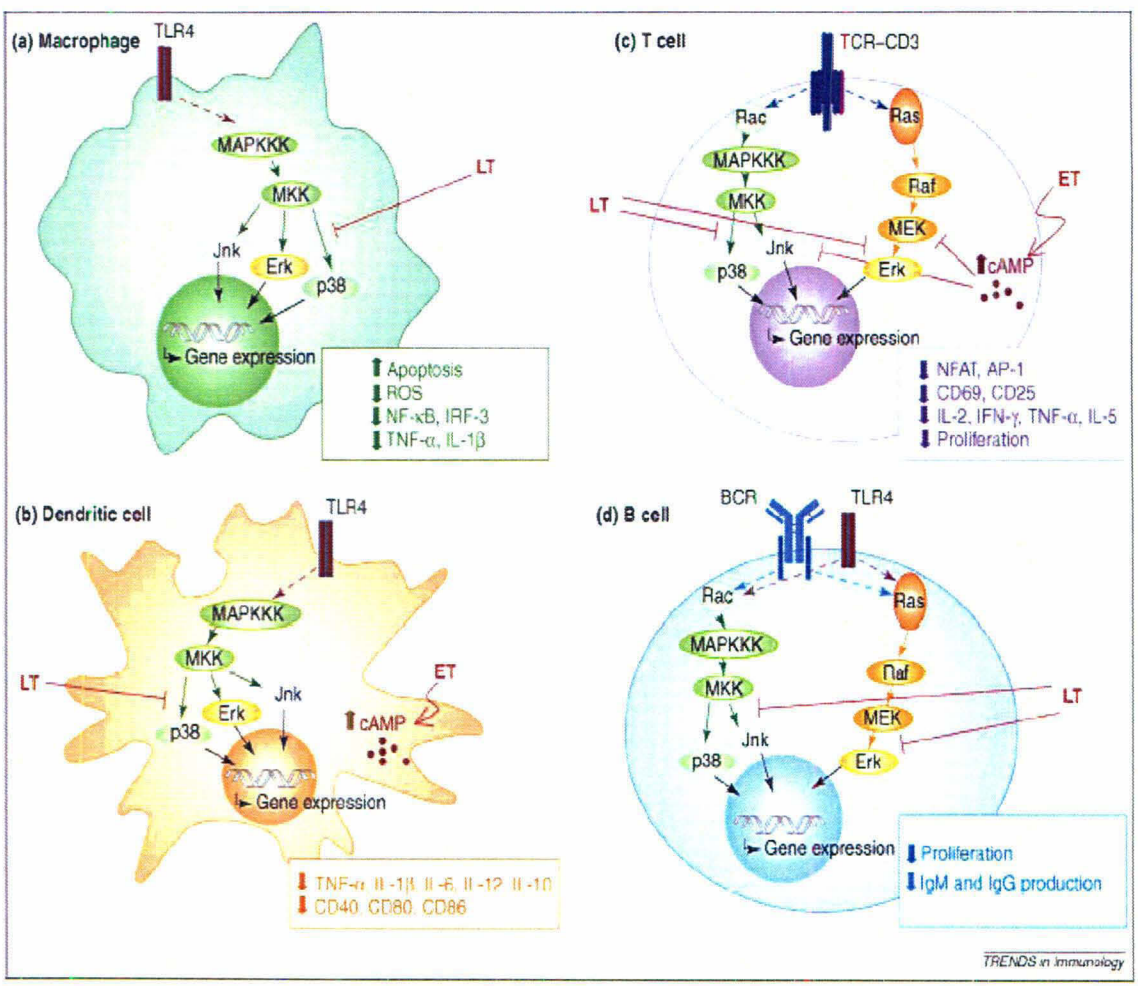


Figure 10. Effects of the anthrax toxins on innate and adaptive immune cells. [Adopted from Trends Immunol 2006, 27:434-40]

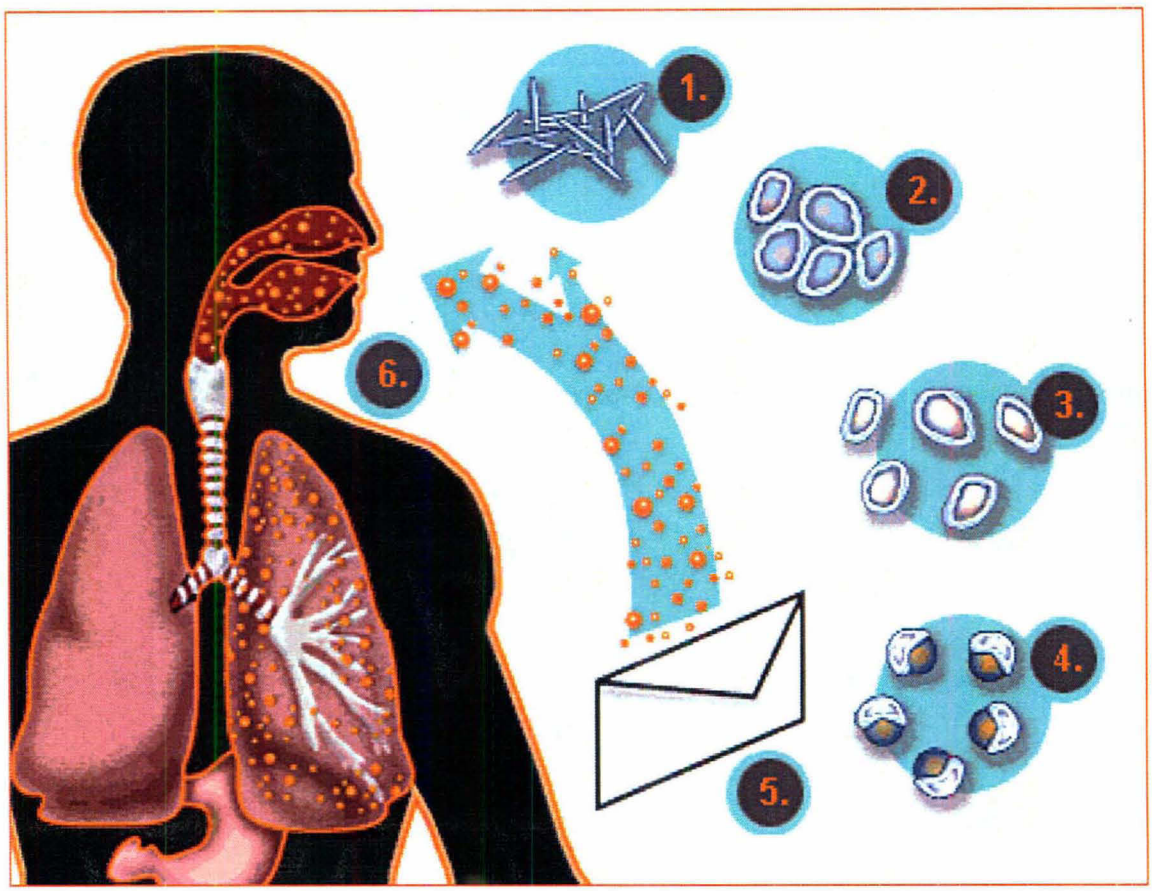
9. THE EFFECT OF ANTHRAX TOXINS ON SPECIFIC IMMUNE CELLS

(Agrawal *et al.*, 2003; Alileche *et al.* 2005; Boyden & Dietrich, 2006; Comer *et al.* 2005; Brittingham *et al.* 2005; Cordoba-Rodriguez *et al.*, 2004; During *et al.*, 2005; Erwin *et al.*, 2001; Fang *et al.*, 2005, 2006; Hsu *et al.* 2004; Kassam *et al.* 2005; Kirby, 2004; Kau *et al.* 2005; O'Brien *et al.* 1985; Paccani *et al.*, 2005; Pellizzari *et al.*, 1999; Park *et al.* 2002; Voth *et al.* 2005; Tournier *et al.* 2005; Warfel *et al.*, 2005; Wu *et al.* 2003)

Table 1. Cellular targets and effects of anthrax toxin

Toxin	Target cell	Effect
Lethal toxin	Neutrophil	Inhibits mobility
	Monocyte	Inhibit proliferation and differentiation
	Activated macrophage	Causes cell death
	Macrophage	Suppresses cytokine production
	Immature dendritic cell	Causes cell death
	Mature dendritic cell	Suppresses cytokine production, co-stimulatory molecule expression and T-cell stimulation
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression
	Red blood cell	Causes cell death
	Platelet	Induces coagulopathy
	Endothelial cell	Causes cell death; promotes cytokine mRNA degradation; dysregulates barrier function
Oedema toxin	Neutrophil	Inhibits phagocytosis
	Macrophage	Causes cell death
	Mature dendritic cell	Suppresses cytokine production
	T cell	Inhibits activation, proliferation, surface-molecule expression and cytokine expression
	Platelet	Induces coagulopathy

10. BIOTERRORISM CAUSED BY *Bacillus anthracis*



Taken at face value, the use of vaccines to prevent the effect of serious infections caused by a terrorist attack appears a sensible policy. In 1997 the United States Department of Defense initiated the compulsory anthrax vaccine immunization programme to immunize 2.4m military personnel. United States military personnel engaged in military operations in Iraq were immunized against anthrax. As in any vaccination campaign, the incidence of the target disease and the characteristics of available vaccines are two key elements in decision making.

Naturally occurring anthrax is a rare disease. It occurs mostly in cutaneous form among those exposed to animal products (such as hides) and causes a rare and rapidly fatal—if untreated—respiratory illness (inhalation anthrax). Inhalation anthrax is the most likely form of the disease in the event of a terror attack as the use of anthrax spores for terror or warfare would probably follow dissemination at high concentration by aerial route. The only recent recorded use of an infectious agent in a terrorist role (the anthrax mailing campaign in the United States in September,

2001) revealed several gaps in the public health system's preparedness against bioterrorism.

There are many potential human biological pathogens that could be used as biological weapons. Upon examining the long list of agents, categorizing each by ease of culture, means of distribution, possible routes of infection, clinical manifestations, and potency, *B. anthracis* is placed very high on this list (Category A) and can very well to be used as a biological weapon. The first and most important characteristic of this organism is its ability to produce small stable endospores. Because the spores are resistant to environmental degradation, they can be used in virtually any climate, contaminating the ground, livestock, and inhabitants for years. The small size of the spores, 2-6 microns in diameter, provides a perfect substance for aerosolization, and is an asset for optimizing inhalation infection (Cieslak 1999). The organism is not fastidious, except for its anaerobic requirement, thus making *B. anthracis* relatively easy to culture on normal media. The clinical signature of inhalation anthrax also aids in the organisms efficiency as a biological weapon. Inhalation anthrax begins with non-specific symptoms of malaise, fever, and a non-productive cough similar to that of a common cold. After a period of 2-3 days, however, symptoms become more severe as the window for possible treatment decreases. This relative lack of an infectious signature until several days after exposure would allow a clean escape for those that release the agent. Contrary to most biological agents, highly sophisticated equipment is not required and the equipment that is required is usually not prohibited under arms control agreements (Siegrist 1999).

While all these characteristics make anthrax a seemingly perfect biological weapon, there are several characteristics that end up working in favor of those protecting themselves from attack. The estimated LD_{50} of 8,000 – 10,000 spores (Meselson *et al.*1994) is rather high and may explain the relative low level of naturally occurring infections in areas of the West with highly contaminated soil. The LD_{50} is also high in comparison to other potential biological weapons such as Q fever and Tularemia (Cieslak 1999).

11. PROPHYLAXIS OF ANTHRAX

Vaccines have proven to be one of the most successful public health measures against naturally acquired infectious diseases; it is no surprise that a top biodefense priority is to develop vaccines against weaponized microorganisms. The performance requirements of biodefense vaccines, however, are somewhat different from those for conventional vaccines. Some key features of biodefense vaccines to be developed for civilian use include (*i*) the rapidity by which a protective immune response can be elicited; (*ii*) the degree to which the vaccine, when administered post-exposure, improves the clinical course of an exposed person; (*iii*) a very high benefit-to-risk ratio of vaccination in all segments of the population; (*iv*) the speed and ease of manufacture, distribution, and administration; and (*v*) the inherent stability of the formulated, final filled product to allow for long-term stockpiling. None of the currently licensed vaccines against select bioterrorism agents meet all of these performance requirements, including the currently licensed anthrax vaccine: "The current anthrax vaccine is difficult to standardize, is incompletely characterized, and is relatively reactogenic (probably even more so because it is administered subcutaneously), and the dose schedule is long and challenging. An anthrax vaccine free of these drawbacks is needed, and such improvements are feasible".

Anthrax vaccines have been in use for a long time. Studies on the vaccination of animals against anthrax date back to the end of the last century. Pasteur demonstrated protective immunization against anthrax in 1881, using a heat-attenuated strain (Pasteur 1881). Although effective, the vaccine suffered from declining potency and troublesome variations in virulence, which occasionally led to the death of animals. The search for a more effective and stable vaccine led to the development of the Sterne attenuated spore vaccine. This vaccine is based on an avirulent non-encapsulated strain called 34F2 derived from the subculture of an isolate from a case of bovine anthrax. Since its introduction, the vaccine has proved safe and extremely effective, and has required little modification. While the vaccine is effective, repeated vaccinations are required for long-term protection; a single dose provides immunity for about a year (Turnbull 2000). Research has shown that the Sterne vaccine has the potential to be developed as a multi-agent animal vaccine platform.

11.1. HUMAN VACCINES

Immunization of humans with live spores has been limited to the former USSR and China (Knop and Abalakin 1986; Dong 1990). Western nations, such as the UK and the United States use non-living subunit vaccines, based primarily on PA, owing to concerns over the possibility of residual virulence (Turnbull 2000).

11.1.1. First-generation human anthrax vaccines. PA is the principal protective immunogen of the US- and UK-licensed human anthrax vaccines. Its ability to stimulate protective immunity in broad range of animal models, including primates, has been repeatedly demonstrated (Baillie 2001). The vaccine licensed in the United States is produced from a cell-free culture filtrate of anaerobically grown *B. anthracis* strain V770-NP1-R (a non-encapsulated, non-proteolytic variant of a bovine strain isolated in Florida in 1951). This cell-free material, which consists largely of PA, is adsorbed onto aluminum hydroxide (Baillie 2001). The immunization schedule comprises a priming course of six doses followed by yearly booster shots. The UK vaccine, while similar in principle to the US vaccine, is produced from an alum precipitate of the cell-free culture filtrate of a static, aerobic culture of the *B. anthracis* Sterne strain 34F2. The priming schedule comprises four doses followed by annual booster shots. In addition to containing large amounts of PA, the UK vaccine also comprises trace amounts of LF and other bacterially derived, immunogenic antigens, which have been shown to stimulate antibody responses in recipients, and might contribute to protection (Baillie *et al.* 2003, 2004). However, the presence of these additional proteins also account for the reactogenicity seen in some individuals (Turnbull 2000). The vaccine has been reported to cause soreness, redness, itching and swelling at the site of inject. Up to 30% of men and 60% of women who received the US vaccine reported mild local reactions lasting up to a week (Wasserman *et al.* 2003). Severe reactions were rare, occurring in <1% of those vaccinated (http://www.anthrax.osd.mil/).

The need to identify correlates of protection is driven by the scarcity of human inhalational anthrax, which makes it impractical to perform human protection studies with these vaccines. For this reason, the Food and Drug Administration in the United States has decided that any new vaccine against anthrax can be licensed

using protection data from two relevant animal models, supported by well-defined correlates of protection (Food and Drug Administration 2002).

11.1.2. Second-generation PA-based vaccines. Second-generation protein-based vaccines differ from their predecessors in that their composition is fully defined, are free from any adverse effects and are produced from media with no animal-derived products. Ideally, the vaccine formulation would be amenable to large-scale production and storage at room temperature and have undergone safety, efficacy and clinical trials. As a consequence, there have been numerous attempts to develop high-level PA expression systems based on a variety of organisms: attenuated strains of *B. anthracis, Bacillus subtilis, Bacillus brevis, and Baculovirus and Escherichia coli* (Coulson *et al.* 1994; Gupta *et al.* 1999; Baillie 2000). Currently, at least two rPA-based vaccines – one produced from an asporogenic variant of the Sterne strain by Vaxgen in the USA and the other from *E. coli* manufactured by Avecia in the UK, are undergoing animal efficacy studies and US National Institute of Health sponsored human safety and immunogenicity trials in the United States. The Vaxgen system, developed by researchers at the US Army Medical Research Institute of Infectious Disease in Frederick, Maryland, comprises a multicopy plasmid-based expression system, which expresses higher levels of PA than the Sterne strain, from which it is derived (Friedlander *et al.* 2002). Also, genetically engineered protein molecules like mutant versions of PA (Chauhan & Bhatnagar 2002; Ahuja *et al.* 2003), LF (Gupta *et al.* 2001) and EF (Kumar *et al.* 2001, Gupta *et al.* 2007) have been used for therapeutic purposes in addition to being exploited as vaccine candidates.

While preliminary animal testing has shown that both the recombinant PA, LF and EF and their mutants require fewer doses than the current licensed vaccines to achieve protection, they still suffer from the need to be given by needle, which requires the involvement of trained medical personnel (Pittman *et al.* 2002; Williamson *et al.* 2005). In addition, the vaccines will have to be transported and stored at 4°C, making it expensive to stockpile in remote regions and limiting its shelf life. Finally, there is still a question regarding the ability of these experimental vaccines to protect humans against inhalational anthrax, given that their efficacy is based on animal data owing to the scarcity of the disease in nature.

11.1.3. Third-generation anthrax vaccines. User-friendly anthrax vaccines which are capable of self administration via the oral, nasal or dermal routes, and induce rapid immunity, following a single dose, in addition to being stable at room temperature, would be extremely attractive to authorities seeking to build stockpiles to respond to a large-scale future threat. To date, efforts to address this requirement have focussed on developing needle-free formulations of PA. Oral vaccines are proven to be an extremely effective means of immunizing large numbers of at-risk individuals, and have been used for many years to confer protection against diseases, such as polio and typhoid (Bhan *et al.* 2005; Kew *et al.* 2005). Attempts to develop an oral anthrax vaccine have focussed on two main approaches: live attenuated bacterial vectors and plant-expressed, edible antigens.

Attenuated *Salmonella enterica serovar typhimurium* has been used as a vehicle for the delivery of heterologous vaccine antigens, and has been shown, in human clinical trails, to have acceptable safety and immunogenicity profiles (Garmory *et al.* 2002). A strain of *S. enterica typhimurium* expressing PA was found to protect mice from lethal anthrax spore challenge (Garmory *et al.* 2003). Unfortunately, it required repeated intravenous dosing, and was ineffective when given orally. *Lactobacillus casei* has also been examined as a potential oral anthrax vaccine (Zeger *et al.* 1999). The bacterium, a commensal of the gut and a food-grade organism, possesses intrinsic adjuvanticity. While studies demonstrated the ability of the organism to express PA, the immune responses in mice were disappointing. Considerable additional research will be required if either of these approaches are to yield a vaccine.

The successful use of plants as expression systems for pathogen antigens, such as PA and human antibodies targeting PA, has opened new possibilities for vaccine and antibody production (Hull *et al.* 2005; Koya *et al.* 2005; Stoger *et al.* 2005). Reports also describe the expression of PA in nuclear transgenic tomato plants, which may serve as a source of an edible vaccine against anthrax (Aziz *et al.* 2001). PA expressed in transgenic tomato plants was able to generate a population of neutralizing antibodies, although at low titer values. These titer values were improved by increasing the expression levels of PA. For this reason, chloroplast transformation technology was used, and increased expression levels of PA in an experimental crop, tobacco, were recorded (Aziz *et al.*2001 & 2005). Plant-based

products are therefore, particularly attractive as they are easily scalable, require little (if any) manufacturing capability, are free of animal pathogens and, in the case of vaccines, have been shown to stimulate immune responses in humans following oral dosing (Tacket *et al.* 2000). While progress in this area is promising, there is as yet no licensed plant-produced human vaccine, and it is likely to be several years before one becomes available.

11.1.4. FOURTH GENERATION ANTHRAX VACCINES

"DNA VACCINES: CRAFTED FROM THE GENETIC MATERIAL"

The concept of vaccination was demonstrated more than 200 years ago when Edward Jenner administered cowpox material to James Phipps and showed that prior exposure to cowpox could prevent infection by smallpox. Over the last century, the development and widespread use of vaccines against a variety of infectious agents have been a great triumph of medical science. Current vaccines target only a tiny fraction of infectious diseases, since prophylaxis against some of the most common and deadly infections in the third world are limited by expense and ease of distribution. In addition to the public health concerns of expense and distribution, other features of current vaccines limit their efficacy. While most current vaccines typically elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex [MHC] class l-restricted cytotoxic T cells) are generally absent or weak. Another limitation of most current vaccines relates to the limited duration of immunologic memory. Ideal vaccines would provide lifelong prophylaxis, a goal generally not achieved by current formulations.

In the past few years, DNA vaccines have burst onto the scene as a radically new approach to infectious disease prophylaxis. The demonstration that plasmid DNA vaccines can induce both humoral and cellular immune responses in a variety of murine and primate disease models has engendered considerable excitement in the vaccine community. The historical basis for DNA vaccines rests on the observation that direct in vitro and in vivo gene transfer of recombinant DNA by a variety of techniques resulted in expression of protein. These approaches included retroviral gene transfer, using formulations of DNA with liposomes or proteoliposomes (Nicolau *et al.* 1983; Kaneda *et al.* 1989; Mannino *et al.* 1988), calcium phosphate-co-precipitated DNA (Benvenisty *et al.*1986), and polylysine-glycoprotein carrier complex (Wu *et al.* 1988). In the seminal study by Wolff et al of "plasmid or naked" DNA vaccination in vivo, it was shown that direct intramuscular inoculation of plasmid DNA encoding several different reporter genes

could induce protein expression within the muscle cells (Wolff *et al.* 1990). This study provided a strong basis for the notion that purified/ recombinant nucleic acids (''naked DNA'') can be delivered in vivo and can direct protein expression. One of the most surprising and important features of DNA immunization is that purified "naked" DNA appears to be taken up and expressed by cells in vivo with much greater efficiency than would have been predicted by the experience with DNA transfection in tissue culture. This finding provides the basis for a critical pharmaceutical advantage of DNA vaccines: namely, simplicity of preparation. In addition, naked DNA can be produced in large scale with tremendous purity, allowing for freedom from contamination with potentially dangerous agents. These observations were further extended in a study (Tang *et al.* 1992), where it was demonstrated that mice injected with plasmid DNA encoding hGH could elicit antigen-specific antibody responses. Subsequently, it was shown that DNA vaccines could protect mice or chickens, respectively, from influenza infection provided a remarkable example of how DNA vaccination could mediate protective immunity (Ulmer *et al.* 1993; Robinson *et al.* 1993). Further studies documented that both antibody and $CD8^+$ cytotoxic T-lymphocyte (CTL) responses were elicited (Ulmer *et al.* 1993), consistent with DNA vaccines stimulating both humoral and cellular immunity.

Thus far, DNA vaccination might provide several important advantages over current vaccines (Table 2). (*a*) DNA vaccines mimic the effects of live attenuated vaccines in their ability to induce major histocompatibility complex (MHC) class I restricted $CD8^+$ T-cell responses, which may be advantageous compared with conventional protein-based vaccines, while mitigating some of the safety concerns associated with live vaccines. (*b*) The final pharmaceutical advantage of DNA is its tremendous stability relative to proteins and other biologic polymers, a feature likely to be more relevant for the production of vaccines than the recreation of dinosaurs. DNA vaccines can be manufactured in a relatively cost-effective manner and stored with relative ease, eliminating the need for a ''cold chain'' (the series of refrigerators required to maintain the stability of a vaccine during its distribution). Hence, this novel class of vaccines, based on immunization with ''naked'' DNA may hold the promise of protecting against human disease without the disadvantages

associated with vaccines presently used, and may help to prevent infections which are not curable today.

TABLE 2 Comparative analysis of various vaccine formulations

		DNA vaccine	Live attenuated	Killed/protein subunit
Immune response				
Humoral	B cells	+++	+++	+++
Cellular	$CD4^+$	+++Th1[a]	+/−Th1	+/− Th1
	$CD8^+$	++	+++	−
Antigen presentation		MHC class I & II	MHC class I & II	MHC class II
Memory	Humoral	+++	+++	+++
	Cellular	++	+++	+/−
Manufacturing				
	Ease of development and production	++++	+	++
	Cost	+++	+	+
	Transport/Storage	+++	+	+++
Safety		+++[b]	++[c]	++++

[a]Th2 responses can be induced by gene gun immunization in mice.
[b]Data available only from Phase 1 trials.
[c]Live/attenuated vaccines may be precluded for use in immunocompromised patients and certain infections such as HIV.

11.1.4. A. DESIGN OF DNA VACCINES

An ideal vaccine would be safe, elicit the appropriate cellular, humoral, or mucosal immunity required for protection, provide long-lived protection at low dose after a single exposure, be easy and inexpensive to manufacture, and be stable when stored or transported under non-optimal conditions. It is unknown whether all of these properties may be incorporated into the design of a DNA vaccine (or any other type of vaccine), but certain of them should be attainable. Furthermore, since the technology for DNA vaccine design and production is generic, these properties may be the same for all DNA vaccines.

11.1.4. A1. VECTOR DESIGN

11.1.4. A1.1. Backbone. Vaccine safety and ease of commercial production initially can be addressed in the design of a standard vector to be used for DNA vaccines. Commercially available vectors, such as those derived from pUC or pBR322, contain only an *Escherichia coli* origin of replication (ORI), thereby allowing them to replicate in *E. coli* for commercial production. They do not contain a mammalian OR1 for replication in mammalian cells. The initial findings by Wolff *et al.* (1990 & 1992a) demonstrated that vectors such as those derived from pUC or pBR322 did not replicate in mammalian cells in vivo at a detectable level. The use of plasmids that do not replicate in mammalian cells should reduce the risk of plasmid integration into the genome. Commercially available vectors have been used extensively for over a decade and their stability and productivity in *E. coli* are well-defined. The copy number of pUC plasmids is 500-700 copies per bacterial cell, while the copy number of pBR322 is 150-200 copies per bacterial cell (Minton *et al.*, 1988). Since pUC based vectors yield more plasmid per bacterial cell, these vectors have been the basis of many DNA vaccines (Manthorpe *et al.* 1993; Montgomery *et al.* 1993; Shiver *et al.* 1995; Freeman and Niven, 1996). Other DNA vaccines have been based on pBR322 vectors (Horn *et al.* 1995). All of these vectors replicate to high copy number throughout the growth of *E. coli* in culture. Runaway-replication plasmids have also been developed that allow copy number to be controlled by the temperature of culture growth (Nordstrom and Uhlin, 1992). These vectors can be kept at a low copy number during cell growth, and then amplified by

shifting the culture to 40°C when the cell mass is high. The copy number of runaway replication plasmids can be as high as 1000 copies per cell. DNA vectors based upon these plasmids would be especially useful for those foreign genes that lower the copy number of pUC-based vectors, such as by low-level production of a plasmid product that is toxic to the bacteria.

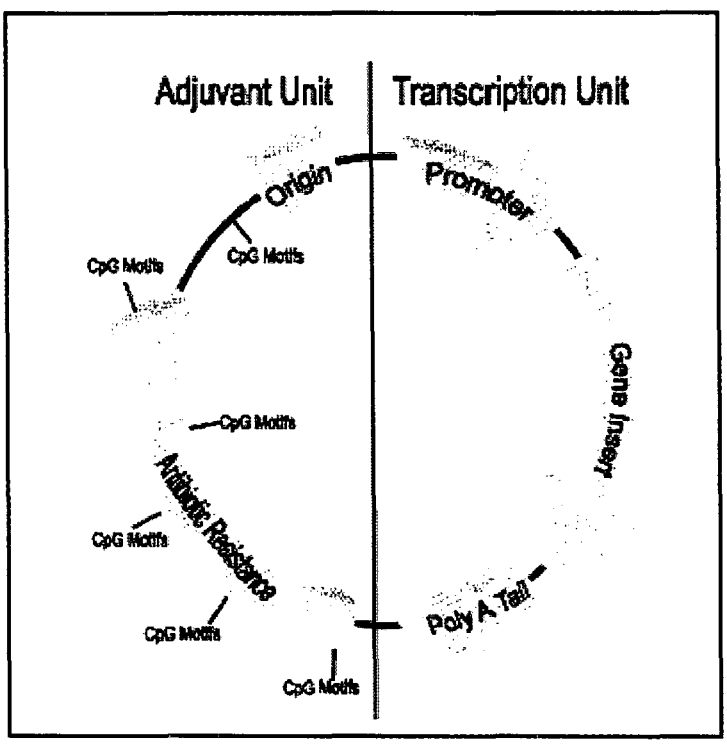


Figure 11. Schematic for the basic requirements of a plasmid DNA vector. The essential features for a plasmid DNA vector include a transcriptional unit, which consists of a viral promoter (i.e. cytomegalovirus), an insert containing the antigen, and transcription/termination sequences (Poly A). The other essential components include a bacterial origin of replication and antibiotic resistance gene, allowing for growth and selection in bacteria. The adjuvant properties of a plasmid vector are highly influenced by the number of CpG motifs within the plasmid backbone. [Adopted from Annu Rev Immunol, 2000, 18: 927-74]

11.1.4. A1.2. Choice of mammalian promoter/terminator. The amount of plasmid that is internalized in vivo has been estimated to be in the picogram range (Wolff *et al.* 1990) after injection into mouse muscle and in the picogram to femtogram range in tissues 1-7 days after intravenous delivery of DNA

complexed with cationic lipids (Lew *et al.*, 1995). Since the plasmids apparently do not replicate, the amount of plasmid available for expression is very low. For this reason, a strong mammalian promoter/terminator should be chosen to drive expression of the antigen gene. Viral gene promoters are usually stronger than cellular housekeeping gene promoters and have been shown to give higher levels of reporter gene expression in vivo after injection into mouse muscle (Manthrope *et al.* 1993). Several groups have seen that the promoter and enhancer from the cytomegalovirus (CMV) immediate early gene 1 yield one of the highest levels of expression (Cheng *et al.*, 1993; Manthrope *et al.*, 1993; Xiang *et al.* 1995). This promoter, along with the CMV intron A, has been used to drive expression of influenza A genes that have elicited protective antibody and immune responses in mice, ferrets, and monkeys (Ulmer *et al.* 1993; Montgomery *et al.* 1993; Donnelly *et al.*, 1995).

Other viral promoters such as the Rous sarcoma virus long terminal repeat (Ulmer *et al.* 1993; Manthrope *et al.* 1993; Xiang *et al.*, 1995), the Simian virus 40 promoter (Cheng *et al.* 1993; Xiang *et al.* 1994) and adenovirus 2 major late promoter (Cheng *et al.* 1993) have been used successfully for gene expression after DNA vaccination, but many laboratories now employ the CMV enhancer and promoter with intron A. Some non-viral promoters may be strong enough to mediate high levels of expression in certain tissues and may allow targeted expression. For example, the albumin promoter and enhancer preferentially expresses genes in the liver (Cheng *et al.* 1993). All of the above promoters give constitutive expression. Few groups have been developing inducible promoters for in vivo regulation of gene expression. The emphasis for this development comes from a need for controlled expression in gene therapy, but inducible promoters may also be useful for some DNA vaccines. For example, there may be a need for vaccines that provide multiple short bursts of antigen production for optimal immune response. Regulated gene expression of certain antigens may also be useful for combination vaccines, where antigenic competition can be a problem, or for vaccines that combine antigen genes with helper genes such as cytokines.

Two examples of inducible promoters based upon drug responsiveness have been shown to function in transgenic mice. Furth *et al.* (1994) have developed a tetracycline controlled transactivating protein that induces expression in the absence

of tetracycline and reduces expression to basal levels in the presence of tetracycline. Liang *et al.* (1996) have reported a similar tetracycline-controlled expression system that functions after injecting mouse muscle with plasmids that express a controllable transactivating factor and a reporter gene. Delort and Capecchi (1996) have developed a promoter that is induced by addition of the drug RU486. In their system, a synthetic steroid receptor must bind to RU486 to activate an inducible promoter that normally does not exist in mammalian cells. The drawback to inducible promoters is the need for additional gene expression to provide the controlling elements. This complicates vector construction for DNA vaccination, but does not preclude its use. Attention should also be paid to the transcription terminator used in conjunction with the promoter. The combination of CMV promoter and bovine growth hormone (BGH) terminator provides very strong transcription. An example of a pUC-based vector containing the CMV/BGH expression cassette is shown in *Figure 11*.

11.1.4. A1.3. Addition of signal sequence. In some cases, it may be desirable to add a mammalian signal sequence for protein secretion to genes from some pathogens. Secretion or localization on a cell surface may be required for efficient antibody production or presentation by MHC Class II molecules, and the native signal sequence may not function well in mammalian cells. An example of a native signal sequence that performs poorly in mammalian cells is that of the *Mycobacterium tuberculosis* secreted protein antigen 85A. Expression and secretion are substantially increased when the mycobacterium signal sequence is replaced by the human tissue-specific plasminogen activator signal sequence (Montgomery *et al.*, 1997).

11.1.4. B. DELIVERY OF DNA

11.1.4. B1. Intramuscular delivery. DNA vaccines have been delivered by a variety of procedures:(1) direct injection of naked DNA, (2) administration of DNA complexed with liposome formulations or other compounds that target specific receptors or compact DNA, and (3) by gold particle bombardment (gene gun administration). Direct injection of naked DNA into skeletal muscle or heart muscle gives better uptake and/or expression than by direct injection into other tissues (Acsadi *et al.*, 1991). It was also found that in mature muscle, naked DNA was superior to certain adenovirus vectors in transferring and expressing reporter genes in muscle fibers (Davis *et al.* 1993). A number of studies have been done to investigate what factors affect the uptake of DNA by muscle cells (Manthorpe *et al.*, 1993; Davis *et al.*, 1994; Wolff *et al.*, 1992a; Levy *et al.* 1996). The connective tissue surrounding myofibers seems to limit the distribution of the DNA to the fibers, thereby limiting the number of transfected cells (Jiao *et al.*, 1992b; Wolff *et al.*, 1992a; Davis *et al.*, 1994). Some researchers have found that regenerating muscle tissue enhances DNA uptake and/or expression (Davis *et al.*, 1994) or that injecting mouse muscles along the longitudinal axis of the muscle and parallel to the myofibers increases uptake and expression (Levy *et al.* 1996). Although the uptake and cellular trafficking of plasmid DNA in muscles is not well understood, Wolff *et al.* (1992a) presented some morphologic evidence that DNA uptake by mouse skeletal muscle may enter through T-tubules and caveolae. Since myocytes have a great number of these structures, this pathway could explain why muscle tissue internalizes naked DNA (i.e., DNA not formulated with cationic lipids or other delivery agents) better than other tissues. Other studies have shown that expression of genes from plasmids after injection into muscles is optimal at 7-14 days post-injection (Manthorpe *et al.*, 1993; Prigozy *et al.*, 1993). Thereafter, expression of reporter genes (such as luciferase) declines, but continues at a low level for the life of the mouse (Wolff *et al.*, 1992b).

11.1.4. B2. Intradermal injection and particle bombardment. Intradermal injection is becoming increasingly popular, as the dense network of antigen-presenting cells in the skin, absent in muscle, provides a favorable

environment for induction of immune responses. This network of langerhans cells (LCs) can aid in the priming of both cellular and humoral immune responses. Intradermal injection has been shown to lead to the transfection of both skin cells and cells with APC-like morphologies (Raz *et al.* 1994). Another method of delivering plasmid DNA to cells is by particle bombardment, whereby DNA is adsorbed onto gold particles and directly delivered intracellularly by high velocity bombardment (gene gun delivery). This method has been used to induce immune responses to human growth factor (Tang *et al.*, 1992), influenza HA (Fynan *et al.*, 1993) and HIV gp120 (Eisenbraun *et al.*, 1993). Gene gun delivery focuses upon delivery to epidermal cells since they are the easiest target to access without surgery. Humoral and cellular immune responses can be achieved with as little as 16 ng plasmid in rodent models and as little as 2-4 pg with larger animals, such as pigs and monkeys (Haynes *et al.*, 1996). Interestingly, it appears that gene gun delivery tends to induce helper T-cell responses of the Th2 phenotype (Haynes *et al.* 1996), whereas i.m. injection of DNA gives a clear Thl type of response. This method has also been used to co-deliver genes for HIV-l gp120 and cytokines to regulate the type of immune response elicited (Prayaga and Haynes, 1996).

11.1.4. B3. Electroporation. Electroporation has also emerged as a promising means by which to increase the potency of DNA vaccines. Following injection of plasmid DNA, electrode needles can be inserted surrounding the site of injection to deliver electric pulses (Aihara *et al.* 1998). This technique has been shown to increase both the number of cells transfected as well as the number of plasmids that enter each cell (Aihara *et al.* 1998). Similar to electroporation in vitro, electric pulses cause a temporary increase in membrane permeability, which allows for the increased passage of plasmid into the cell. Electroporation can be used to deliver plasmid to both muscle and skin, and has been shown to significantly enhance plasmid-induced immune responses in a variety of model antigen and animal systems, including mice, guinea pigs, rabbits, pigs, and rhesus macaques (Babiuk *et al.* 2002; Widera *et al.* 2000). However, concerns regarding electroporation include an increased risk of plasmid integration into the host genome and pain upon delivery. Improvements in probe design and voltage and pulse lengths will be important for advancing this technology.

11.1.4. B4. Complexed DNAs. In an attempt to protect DNA from degradation and to potentially target it to specific tissues, plasmids have been complexed with lipids and with ligands that target cell surface receptors. These complexes are thought to be internalized by endocytosis (Zhou and Huang, 1994; Friend *et al.*, 1996) and hence, must escape the endosome, enter the cytoplasm, and finally enter the nucleus to effectively transfect cells. Addition of fusogenic peptides and other endosome-destabilizing molecules (Mizuguchi *et al.*, 1996) or nuclear proteins that contain nuclear targeting signals (Kaneda *et al.*, 1989) has been shown to increase the expression from such complexed plasmids in tissue culture. Liposome/DNA complexes are internalized and expressed by liver, lung, heart, kidney, and spleen (Stewart *et al.*, 1992; Stribling *et al.*, 1992; Lew *et al.*, 1995). They are also taken up by cells in muscle tissue (Wolff *et al.*, 1992a; Lew *et al.*, 1995), but the rate of uptake and expression in muscle is much less than from naked DNA (Wolff *et al.*, 1992a). Most of the complexes are composed of cationic liposomes. DNA/ liposome complexes enhance the uptake in airway epithelium (McLachlan *et al.*, 1994), and have been shown to produce mucosal antibodies, as well as systemic humoral antibodies to HIV envelope (Mitchell *et al.*, 1996). The preparation of optimal DNA/cationic liposome complexes involves many parameters, such as DNA:lipid ratio during production. DNA-ligand complexes for receptor-mediated internalization have been used successfully to target DNA to specific tissues. The initial work was done to target DNA delivery to the liver for gene therapy (Wu and Wu, 1988). Most ligands are conjugated to a polycation, usually polylysine, for association with negatively charged DNA (Perales *et al.*, 1994).

11.1.4. C. IMMUNOLOGY OF DNA VACCINES

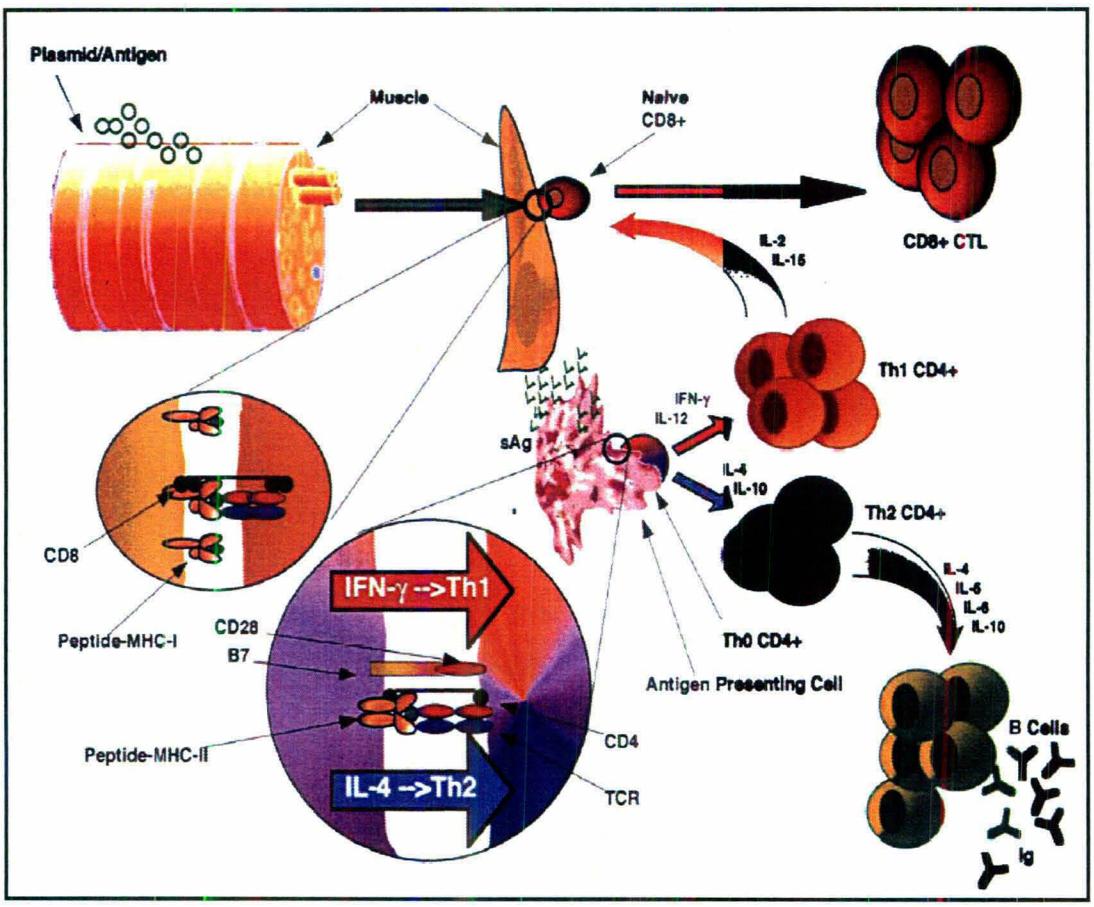


Figure12. Immune responses elicted by plasmid DNA inoculation. The direct intramuscular inoculation of DNA expression cassettes leads in vivo transfection of muscle fibers. Expression of the plasmid-encoded protein (or proteins) may elicit an immune response. Secreted immunogens are ingested by phagocytosis and presented as a peptide-MHC II complex by professional antigen-presenting cells. These cells can provide the primary activation signal, costimulatory ligands, and cytokines necessary to stimulate naive T cells. Stimulation of ThO T cells with IL-4 leads to the development of the Th2 $CD4^+$ helper T cell, which will secrete cytokine to promote B cell development, including IL-4, IL-5, IL-6, and IL-b. Stimulation of ThO cells with the proinflammatory cytokines IL-12 and IFN-y leads to development of the Th1 $CD4^+$ helper T cell. These cells secrete cytokines that will promote the development of $CD8^+$ cytotoxic T lymphocytes. [Adopted from FASEB J, 1997, 11: 753-63]

An important first step in the rational design of a vaccine is to understand the immune correlates of protection. For most viral and bacterial infections, primary protection is mediated by a humoral immune response (production of antibodies). For intracellular infections such as *Mycobacterium tuberculosis, Leishmania major,* and other parasites, protection is mediated by cellular immunity. Moreover, for some

diseases [e.g. human immunodeficiency virus (HIV) infection, herpes, anthrax and malaria], both humoral and cellular responses are likely to be required. The cellular immune response comprises primarily $CD4^+$ and $CD8^+$ T cells. These cells recognize foreign antigens that have been processed and presented by antigen presenting cells (APCs) in the context of MHC class II or class I molecules, respectively *(Figure 12)*. Exogenous antigens provided by killed/inactivated pathogens, recombinant protein, or protein derived from live vaccines are taken up by APCs by phagocytosis or endocytosis and presented by MHC class II molecules to stimulate $CD4^+$ T cells, which can help generate effective antibody responses. In contrast, MHC class I molecules associate with antigens synthesized within the cytoplasm of the cell (with rare exceptions) and are generally elicited by live or DNA vaccines. From an immunologic standpoint, based on the broad range of effector cells generated and the memory responses they induce, live attenuated vaccines represent the vaccines of choice for those diseases requiring both humoral and cellular responses (Table 2). From a practical and safety standpoint, however, live or live attenuated vaccines raise several issues that can preclude their widespread use. In this regard, DNA vaccines—which resemble live attenuated vaccines in their ability to induce both humoral and cellular responses—may prove to be useful alternatives.

Despite the flurry of reports documenting the ability of naked DNA vaccines to induce both immunologic and protective responses in animal models, the mechanism by which DNA injections activate the immune system against the encoded antigens remains somewhat mysterious. Nonetheless, given what is now understood about pathways of antigen processing and the requirements for T cell activation, exposing the mechanisms of immune activation by naked DNA may reveal some provocative clues to how the immune system deals with different forms of antigen. One intriguing aspect of DNA vaccination involves the mechanism by which the antigen encoded by the foreign gene introduced into the bacterial plasmid is processed and presented to the immune system. Studies demonstrate that the quantity of antigen produced in vivo after DNA inoculation is in the picogram to nanogram range. Given the relatively small amounts of protein synthesized by DNA vaccination, the most likely explanation for the efficient induction of a broad-based and sustained immune response is the immune-enhancing properties of the DNA itself (i.e. CpG motifs) and/or the type of APC transfected. There are at least three

mechanisms by which the antigen encoded by plasmid DNA is processed and presented to elicit an immune response: (*a*) direct priming by somatic cells (myocytes, keratinocytes, or any MHC class II-negative cells); (*b*) direct transfection of professional APCs (i.e. DCs); and (*c*) cross-priming in which plasmid DNA transfects a somatic cell and/or professional APC and the secreted protein is taken up by other professional APCs and presented to T cells.

11.1.4. C1. DIRECT TRANSFECTION AND PRIMING OF PROFESSIONAL ANTIGEN-PRESENTING CELLS (APCs)

11.1.4. C1.1. Bone Marrow-Derived Cells Directly Mediate Cellular Immune Responses after DNA Vaccination *(Figure 13 A)* Several elegant studies with bone marrow-chimeric mice have conclusively demonstrated that bone marrow-derived APCs play a key role in the induction of the immune response after DNA vaccination. In these studies, parent into F1 bone marrow-reconstituted mice created a mismatch between the haplotypes of somatic cells and bone marrow-derived cells. The immune response generated on subsequent DNA immunization was found to be restricted to the haplotype of reconstituted bone marrow, providing conclusive evidence that bone marrow-derived cells were responsible for priming immune responses after DNA vaccination (Corr *et al.* 1996; Iwasaki *et al.* 1997; Doe *et al.* 1996). However, the most direct evidence implicating bone marrow derived APCs in the priming events of naked DNA vaccines comes from a study by Ertl and colleagues. Using the murine rabies model, they demonstrated that coinjection of naked DNA encoding murine granulocyte macrophage colony-stimulating factor (GM-CSF) with the gene encoding rabies glycoprotein enhanced both antibody and cellular responses to the rabies glycoprotein antigen (Xiang and Ertl, 1995). The proposed mechanism by which paracrine GM-CSF elaboration enhances antigen-specific immune response relates to the ability of this cytokine to induce the differentiation of hematopoetic progenitors into "professional" APCs. In addition to macrophages, GMCSF has been shown to induce the differentiation and maintenance of dendritic cells. This cell type, which is 2-3 three logs more potent on a per cell basis than macrophages in activating naive T cells in vitro, has been proposed to be the critical APC in initiating immune responses in vivo. Qualitative

and quantitative differences in APC composition or traffic into different tissue types may, in fact, account for the lower amount of DNA necessary for intradermal vaccination. In particular, the presence of Langerhans cells in the epidermis provides a ready source of APCs at the injection site.

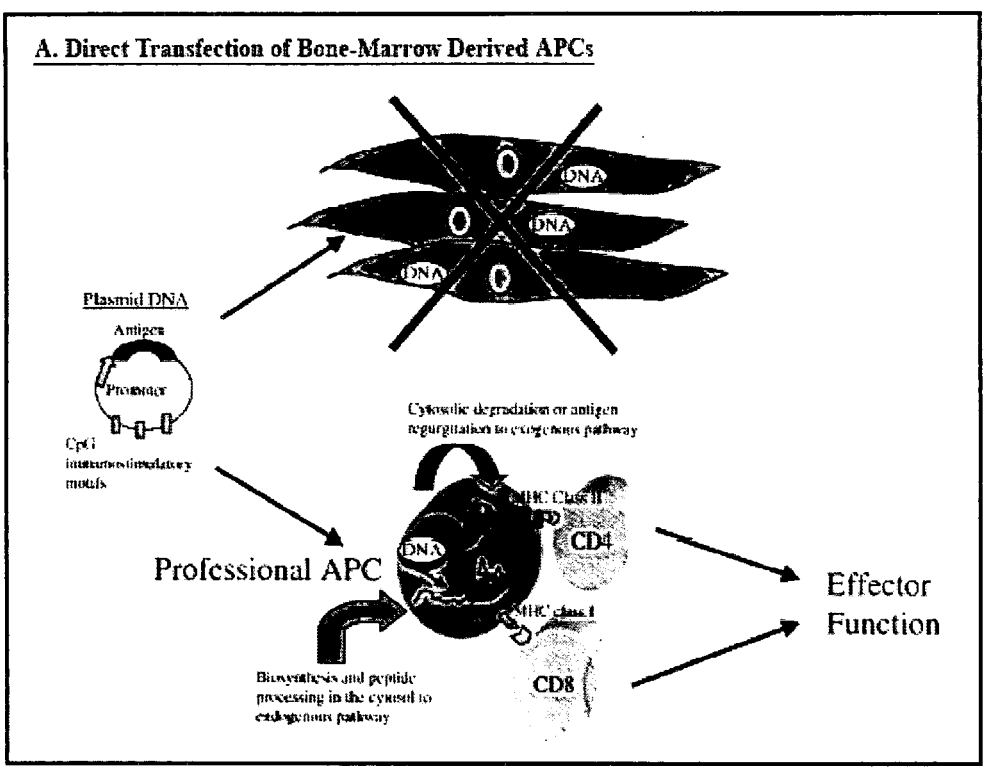


Figure13A. Mechanisms of antigen presentation after DNA immunization. Bone marrowderived antigen-presenting cells (APCs)s mediate immune responses after DNA vaccination. Injection of plasmid DNA leads to direct transfection of a small number of Dendritic cells that present antigen to T cells. [Adopted from Annu Rev Immunol, 2000, 18: 927-74]

11.1.4. C1.2. Dendritic Cells Are the Principal Cells Initiating the Immune Response after DNA Vaccination. Following intramuscular injection of a plasmid DNA vaccine in mice, expression of the encoded antigen occurs primarily in the transfected myocytes at the site of inoculation (Wolff *et al.* 1990). Myocytes lack expression of MHC II and co-stimulatory molecules and thus would not expect to prime T lymphocytes directly. Therefore, studies were aimed at defining the specific type of APCs regulating the immune response after DNA vaccination. The first study to address this question showed that isolated dendritic cells (DCs) but not B cells or keratinocytes from DNA vaccinated mice were able to efficiently present antigen to T cells in vitro (Casares *et al.* 1997). Moreover, in the

same study it was estimated that only a small proportion of the DCs (0.4%) were transfected with plasmid DNA (Casares *et al.* 1997). Similar results were obtained in two additional studies in which the injection of DNA led to direct transfection of small numbers of DCs (Porgador *et al.* 1998; Akbari *et al.* 1999). It is notable that in both of these studies there was general activation and migration of large numbers of DCs that were not transfected.

Finally, direct in vivo visualization of antigen-expressing DCs from draining lymph nodes after gene gun vaccination was demonstrated in a separate study in which gold particles and protein expression from a reporter gene could be co-localized within a cell that had morphologic indices consistent with a DC (Condon *et al.* 1996). Taken together, the preponderance of data clearly demonstrated that DCs play a key role in induction of the immune response after DNA vaccination. Furthermore, these data suggested that the predominant contribution to priming immune responses after DNA vaccination involved a small number of directly transfected DCs. Additionally, as noted above, the question arised whether the enhancement in the number of migrating DCs not directly transfected with DNA, seen in many studies, could also present antigen via additional mechanisms such as cross-priming.

11.1.4. C1.3. Direct Priming of Somatic Cells—Skin vs Muscle. The initial seminal study by Wolff *et al.* (1990) demonstrating the success of ''plasmid or naked'' DNA vaccination in vivo involved the direct intramuscular inoculation of plasmid DNA, leading to expression of protein within the transfected cell *(Figure 13B).* Another important study (Ulmer *et al.* 1993) showed that direct intramuscular inoculation of plasmid DNA induced a strong $CD8^+$ CTL to influenza nucleoprotein, provided the first evidence that cellular responses could be induced in vivo and have a potentially important protective role. These and several additional studies suggested that muscle cells were critically involved in the initiation of immune responses after DNA vaccination. One conceptual difficulty with this premise was that, although muscle cells express MHC class I molecules, they do not express other cell surface molecules (i.e. CD80 and CD86) that are critical in optimizing T-cell priming.

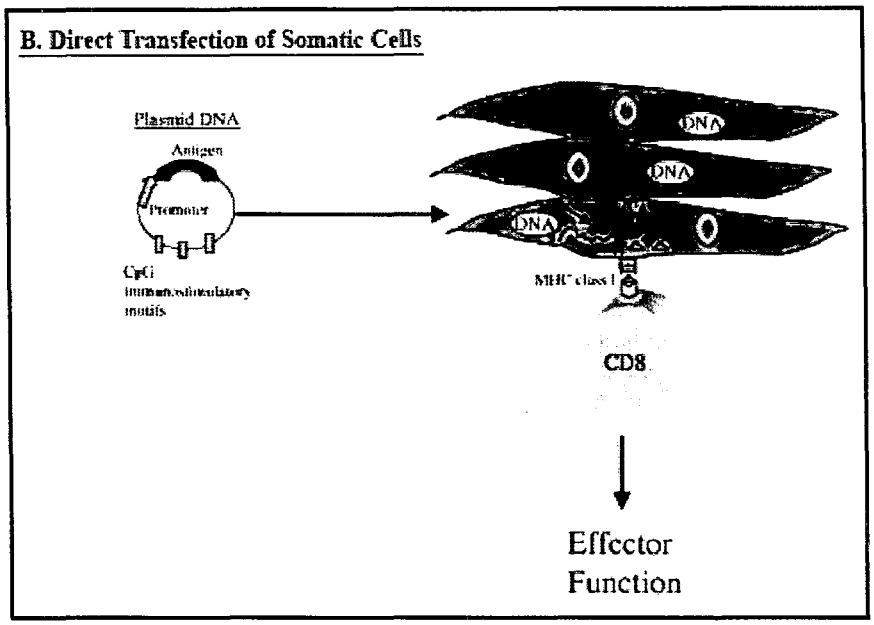


Figure 13B. Mechanisms of antigen presentation after DNA immunization. Direct priming of immune responses by somatic cells (myocytes, keratinocytes, or any major histocompatibility complex class-II–negative cells). This result could occur after injection of plasmid DNA into muscle or skin, leading to protein production and presentation to T cells by the somatic cells themselves. [Adopted from Annu Rev Immunol, 2000, 18: 927-74]

Therefore, they are not likely to be as efficient at presenting antigen as are DCs. This difficulty raised a question about the exact role that muscle cells play in the induction of cellular immune responses after intramuscular DNA vaccination. To address whether expression of antigen by myocytes was sufficient to induce protective immunity in vivo, it was shown that transfer of stably transfected myoblasts expressing an influenza nucleoprotein protected mice from infectious challenge (Ulmer *et al.* 1996). Although these data suggested that expression of viral protein by muscle cells in vivo is sufficient for CTL-mediated protection, the question of whether CTLs were induced directly by myocytes expressing protein directly or by transfer of protein from myocytes to professional APCs (cross-priming) remained open.

Experiments were undertaken to directly test whether muscle cells alone are sufficient to prime immune responses. In one study, using bone marrow chimeras to examine the contribution of bone marrow- and non-bone marrow–derived cells to CTL priming, it was shown that antigen-specific CTL responses could be induced by non-bone marrow–derived (muscle) cells only when mice were vaccinated with DNA encoding the antigen and CD86 (Agadjanyan *et al.* 1999). By contrast, in a

separate study with a plasmid DNA encoding a different antigen, it was shown that plasmids encoding CD86, IL-12, or granulocyte/macrophage colony-stimulating factor DNA failed to induce muscle cells to prime for CTL responses (Iwasaki *et al.* 1997). Taken together, although these studies both show that muscle cells alone are not efficient at priming immune responses, one study does suggest that muscle cells expressing CD86 are sufficient to induce a response. Finally, the finding that removing the muscle immediately (within 10 min) after immunization does not alter the subsequent immune response (Torres *et al.* 1997) provides additional evidence that injected plasmid DNA is likely to gain access to the lymphatic or circulatory system, thus obviating the need for transfection of muscle cells at the site of injection. For other somatic cells, it has been shown that keratinocytes and Langerhans cells constitute the major cell types transfected by plasmid DNA after injection into the skin (Yang *et al.* 1990; Raz *et al.* 1994). In contrast to the data mentioned above regarding removing muscle, immediate removal of skin after DNA vaccination prevented development of immune responses (Torres *et al.* 1997). Moreover, in a separate study, it was shown that transplantation of vaccinated skin, 12 h post vaccination could elicit an immune response in naive animals (Klinman *et al.* 1998). By contrast, little or no immune response could be initiated when the period of transplantation exceeded 24 h. These data suggest that cells that migrated from the epidermis within 24 h of immunization induced the primary immune response after DNA vaccination. Finally, it was shown that the magnitude of the primary immune response increased when the vaccination site was left intact (Klinman *et al.* 1998). Taken together, these data suggest that antigen-expressing non-migratory cells such as keratinocytes may continue to produce antigen to augment the immune response (Akbari *et al.* 1999; Klinman *et al.* 1998).

11.1.4. C1.4. Cross-Priming. Secreted or exogenous proteins undergo endocytosis or phagocytosis to enter the MHC class II pathway of antigen processing to stimulate $CD4^+$T cells. Endogenously produced proteins/peptides (e.g. viral antigens) are presented to the immune system through an MHC class I dependent pathway to stimulate naive $CD8^+$ T cells. Although peptides derived from exogenous sources are generally excluded from presentation on MHC class I molecules, there are now several examples showing that this can occur in vivo

(Wraith *et al.* 1987; Staerz *et al.* 1987; Harding *et al.* 1994). Moreover, the concept of cross-priming, in which triggering of $CD8^+$ T-cell responses can occur without de novo antigen synthesis within the APCs, provides an additional mechanism by which DNA immunization can enhance immune responses. During cross-priming, antigen or peptides (both MHC class I and II) generated by somatic cells (myocytes or keratinocytes) can be taken up by professional APCs to prime T-cell responses *(Figure 13C).* The demonstration that transfer of myoblasts expressing an influenza nucleoprotein into F1 hybrid mice induced CTL responses restricted by the MHC haplotype of the recipient mice provided the first evidence that transfer of antigen from myocytes to professional APCs can occur in vivo in the absence of direct transfection of bone marrow–derived cells (Ulmer *et al.* 1996; Fu *et al.* 1997).

In addition, cross-priming can occur when professional APCs process secreted peptides or proteins from somatic cells and/or other APCs by phagocytosis of either apoptotic or necrotic bodies (Albert *et al.* 1998 a & b). This is supported by a study showing that cross-priming of DCs occurred when keratinocytes expressing antigen were exposed to irradiation in vitro, leading to cell death (Akbari *et al.* 1999). In summary, the overwhelming evidence suggests that bone marrow-derived APCs, but not somatic cells, directly induce immune responses after DNA vaccination; however, because somatic cells such as myocytes or keratinocytes constitute the predominant cells transfected after DNA inoculation via muscle or skin injection, respectively, these cells may serve as a reservoir for antigen. Thus, somatic cells can be important in the induction of immune responses via cross-priming and may play a role in augmenting and/or maintaining the response.

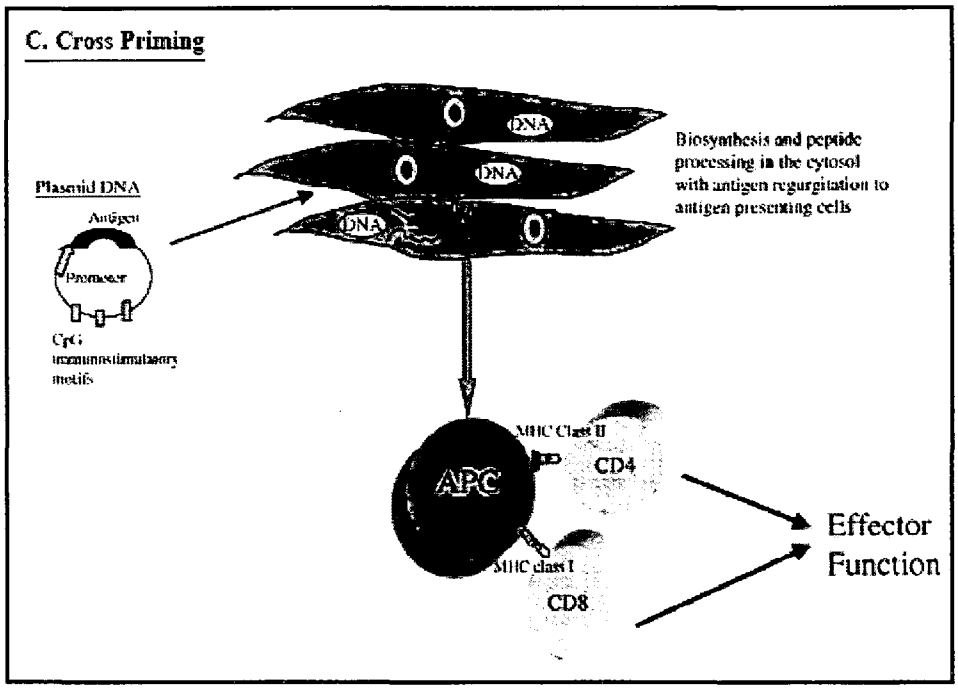


Figure 13C. Mechanisms of antigen presentation after DNA immunization. Protein production by transfected somatic cells may be taken up by professional APCs, leading to T-cell activation (cross-priming). [Adopted from Annu Rev Immunol, 2000, 18: 927-74]

11.1.4.D. POTENTIAL WAYS TO ENHANCE THE POTENCY OF DNA VACCINES

Conceptually, there are three ways in which DNA vaccines may be improved: by modifying the vector to maximize expression or immunogenicity; by formulating the vector to optimize delivery to and transfection of cells in vivo; and by the inclusion of adjuvants to enhance or modify immune responses against the expressed antigen. The following is a discussion of various ways that have been shown to or have the potential to increase the potency of DNA vaccines.

11.1.4. D1. Expression. Plasmid DNA vectors that are developed for vaccination contain certain important features, such as a strong promoter/enhancer, a polyadenylation termination sequence, an origin of replication for the production of the plasmid in *Escherichia coli,* an antibiotic resistance gene as a selectable marker, and convenient cloning sites for the insertion of genes of interest. A basic premise governing the development of vectors for vaccination purposes is that a high level of protein expression is desirable, that is, higher expression in vitro equates with higher expression in vivo, which in turn results in stronger immune responses. It is likely that this will prove to be generally true, although there may be mitigating factors that can complicate this relationship. As examples, high levels of a protein that is toxic to cells could limit the duration of expression, and the expression of a protein that has effects on other cells (including cells of the immune system) could decrease immune responses directed against it. In addition, the modification of vectors could inadvertantly introduce specific nucleotide sequences that have modulatory effects on immune cells (Sato *et al.* 1996). Specific modifications to a vector that could increase overall protein expression include the following: the particular combination of promoter and terminator (Montgomery *et al.* 1993; Liang *et al.* 1996); the removal of elements not essential for eukaryotic expression, such as those contained in multi-purpose expression vectors; the inclusion of enhancer elements such as the intron A region of the cytomegalovirus promoter (Chapman *et al.* 1991); and elements that confer mRNA stability.

In some cases, it may be desirable to have an inducible promoter to introduce a degree of control over expression *in vivo*. Such plasmids have been designed to be sensitive to tetracycline which, depending on the construct, has the potential to act in a repressive or activating fashion (Liang *et al.* 1996; Dhawan *et al.* 1995). Manipulation of plasmid DNA vectors to achieve increased levels of expression *in vivo* is a laudable goal of DNA vaccine development. This approach is particularly attractive since changes to the sequence of the vector do not affect the formulation of the vaccine itself, unlike other potential approaches that involve additional components, thereby increasing the complexity of the vaccine.

11.1.4. D2. Protection from degradation. Extracellular spaces in tissues provide a harsh environment for some biological molecules and unprotected DNA is rapidly degraded. In vitro, supercoiled plasmid DNA is converted to linear DNA then to small molecular mass fragments within minutes upon the addition of serum (D Montgomery, JB Ulmer, MA Liu, unpublished data). In vivo, plasmid DNA was also found to be rapidly degraded when analyzed within minutes of injection into mouse muscle (Wolff *et al.* 1991). This lability is particularly relevant for i.m. injection of DNA, which presumably requires an active DNA uptake process. Hence, the time between deposition of DNA in the tissues and cellular uptake is a critical period in determining the amount of DNA available for transfection of cells. Therefore, an effective barrier between the DNA and extracellular nucleases may be a valuable means of increasing the potency of DNA vaccines. Some potential ways that DNA could be protected from degradation in situ include encapsulation in liposomes or microspheres, or compacting the DNA into small particles, such as can be accomplished with certain polymers. However, even cationic liposome/DNA formulations, which can provide protection from degradation of DNA in vitro (Wasan *et al.* 1996), have been shown to have a very short half-life (~5 min) in vivo after intravenous injection (Lew *et al.* 1995). A potential complication in identifying an effective formulation that stabilizes DNA in vivo is that it must also be compatible with other aspects of the transfection process. Ideally, such a formulation should protect the DNA and enhance cellular uptake and targeting to the nucleus and then dissociate so as not to interfere with transcription.

11.1.4. D3. Distribution in muscle. In skeletal muscle there are significant impediments to the free diffusion and dispersion of injected fluids. These include, most notably, the connective tissues that surround fiber bundles and provide tensile strength to the muscle. Therefore, it is possible that many myocytes never come into contact with injected fluids and, hence, have little chance of being transfected with DNA vaccines. Indeed, histochemical and immunochemical analyses of muscle tissues injected with DNA encoding bacterial reporter proteins or viral antigens, respectively, have shown that only a small fraction of myocytes are detectably transfected and these are generally clustered around the injection site (Wolff *et al.* 1990; Davis *et al.* 1993). If a means of increasing the distribution of injected DNA could be introduced, a greater number of cells could potentially be transfected. Increased protein expression and an increase in the number of myocytes transfected, albeit modest, have been achieved by pretreatment with agents that cause muscle fiber destruction and ensuing muscle regeneration, such as bupivicaine (Wells *et al.* 1993; Vitadello *et al.* 1994) and cardiotoxin (Davis *et al.* 1993), or pretreatment with hypertonic sucrose, which results in an increased volume of extracellular fluid compared to intracellular fluid (Davis *et al.* 1993). These observations could be explained by an increased accessibility of myocytes at the time of DNA injection. However, these increases in protein expression have translated into little, if any, improvements in DNA vaccine efficacy. Moreover, if the lack of distribution of DNA in the muscle were a substantial problem, one would expect that multiple injections throughout the muscle would enhance total protein expression, but this has not been seen (Wolff *et al.* 1991; Manthorpe *et al.* 1993). Therefore, it is unclear how much improvement in vaccine efficacy can be achieved by increasing DNA distribution in muscle and, for this reason, it may be reasonable to focus efforts on the other areas discussed here or target other cell types.

11.1.4. D4. Cellular uptake. The means by which injected DNA is internalized by cells is not known. However, several lines of evidence suggest that there may be a physiological mechanism involved. First, it is theoretically possible that injury to the multinucleated myocytes could result in transient disruptions of the plasma membrane, thereby allowing the entry of extracellular DNA. Yet, it does not appear that this explains uptake after DNA injection, as attempts to specifically

damage muscle have not resulted in enhanced expression (Wolff *et al.* 1991; Manthorpe *et al.* 1993). Second, myocytes are the most easily transfected cells after direct DNA injection (Wolff *et al.* 1990). Other types of cells can also be transfected upon direct injection, but the levels are substantially lower than in muscle, suggesting that there may be a DNA uptake process that is more active or efficient in muscle cells than in other types of cells. Third, a morphologic study to investigate tissue localization of DNA after injection demonstrated the preferential localization of DNA in myocyte membrane invaginations, termed caveolae, and T-tubules (Wolff *et al.* 1992). These observations could be explained simply by a sequestration of DNA in these compartments or by an uptake process of DNA, because these membrane systems may offer a portal into the interior of muscle cells. The lack of understanding of DNA uptake by cells limits the ability to design rational approaches to facilitate this process.

Nevertheless, there are certain avenues that one could logically take. For example, cationic liposomes have been very effective in facilitating the transfection of cells *in vitro* and some formulations may prove to be effective in vivo. So far, however, little progress has been reported on the use of cationic liposomes for i.m. injection of DNA, but transfection of cells has been demonstrated after intranasal administration of DNA/cationic liposome complexes (Stribling *et al.* 1992) and suggests that immune responses may be induced this way. Synthetic polymers, such as polyvinylpyrolidone, have also been used with some success to facilitate gene transfer in vivo (Mumper *et al.* 1996). It is not known how such polymers function, but they compact plasmid DNA and may thereby enhance uptake by endocytosis. Several other compounds have been shown to increase transfection of cells *in vitro,* but their utility in vivo has not been reported. Finally, targeting of DNA to cell-surface receptors has been effective both in vitro and in vivo. Wu and Wu (1988) successfully targeted hepatocytes for uptake by complexing DNA to molecules with affinity to the asialoglycoprotein receptor. In this way, intravenously injected DNA was preferentially internalized by hepatocytes in vivo. In theory, abundant surface receptors on any cell type could be targeted, providing one knew which cell type to target for transfection by DNA vaccines. The i.m. injection of DNA is an effective route of immunization. In fact, for cell-mediated immunity against influenza in mice, i.m. is superior compared to other routes (Donnelly *et al.* 1994). Whether the

transfection of muscle cells is necessary for the induction of robust immune responses is questionable, given the effectiveness of particle bombardment (Fynan *et al.* 1993; Tang *et al.* 1992; Barry *et al.* 1995), which is thought to transfect cells of the dermis and epidermis rather than muscle cells. Nevertheless, the expression of antigens in muscle cells alone is sufficient to induce protective cell-mediated immune responses and there may be certain advantages to the i.m. route (e.g. it is a clinically accepted mode of vaccination, it does not require a device, and it targets terminally differentiated cells). Hence, targeting of myocytes for transfection may be a reasonable strategy. The identification of methods to facilitate DNA uptake by cells is a key area of DNA vaccine development. However, the lack of an in vitro model to test potential DNA formulations poses a significant limitation to predicting how they would perform in vivo and necessitates the time-consuming process of screening DNA vaccine formulations in animal models. Therefore, efforts need to be directed towards an understanding of the mechanisms of cellular uptake of DNA.

11.1.4. D5. Modulation or enhancement of immune responses. Many conventional vaccines are administered with an adjuvant that enhances the immune response against the co-injected antigen. The only adjuvants currently approved for human use are aluminum salts, but there are many experimental adjuvants that have been shown to increase immunogenicity, modulate immune responses or facilitate the induction of CTL responses against co-administered antigens. In theory, these could also be used in combination with DNA vaccines. However, there are several potential complications that apply specifically to DNA vaccines: there may be a lag period between the injection of DNA and the expression of antigen; the expression of the antigen may outlast the presence of the adjuvant; the relevant expression of the antigen may not be at the site of injection, as could occur if mobile cells are transfected or if DNA is carried to other sites in the body; and the potential exists for an adjuvant effect leading to the induction of immune responses against the DNA itself. Therefore, the route and timing of administration of an adjuvant with DNA will need to be explored. The following are two novel approaches that have had a demonstrable effect on the immunogenicity of DNA vaccines.

11.1.4. D6. Cytokines. Other immunomodulatory molecules, such as cytokines, may be effective at enhancing immune responses against proteins expressed in situ by DNA vaccination. For example, an immune response directed preferentially toward a specific T-helper cell response may be achieved through the use of specific cytokines (e.g. interleukin [IL]-2, IL-12 and interferon-γ, for Thl; IL-4 and IL-10 for Th2; and granulocyte-macrophage colony stimulating factor [GM-CSF] that can also exert effects on other cells of the immune system). These cytokines could be delivered as recombinant proteins or could be expressed *in situ* using plasmid DNA (Hengge *et al.* 1995; Irvine *et al.* 1996). In addition, DNA encoding co-stimulatory molecules, such as B7-1 and B7-2, have been used in conjunction with DNA vaccines (Conry *et al.* 1996). Co-administration of plasmids encoding viral (Ziang *et al.* 1995), bacterial (Tascon *et al.* 1996) or tumor (Conry *et al.* 1996) antigens with separate plasmids encoding GM-CSF or B7-1 has been shown to have a modest enhancing effect on antibody titers. These early-stage studies provide encouraging evidence that expression of immunostimulatory molecules in vivo may be useful for enhancing or modulating immune responses induced by DNA vaccines. However, the potential for unregulated expression of a cytokine in vivo may limit the use of such an approach to situations where the benefit:risk ratio is greater (e.g. HIV or cancer immunotherapy) than for prophylactic use in healthy people.

11.1.4. D7. Adjuvanticity of DNA. An ideal means of enhancing DNA vaccines would be to optimize the vector itself, because this would not require complicated vaccine formulations. This optimization could be in the form of increased levels of antigen expression or by incorporating specific nucleotide sequences that have been shown to exert effects on immune cells. It has been known for some time that GC-rich DNA from bacterial sources can stimulate natural killer (NK) cell (Yamamoto *et al.* 1992) and B lymphocyte (Messina *et al.* 1991) activity in vitro. A particular motif consisting of unmethylated purine-purine-C-G-pyrimidine-pyrimidine was subsequently found to be particularly active (Krieg *et al.* 1995) and resulted in secretion of IL-6, IL-12 and interferon-γ (from cells incubated with oligonucleotides *in vitro* (Yi *et al.* 1996; Klinman *et al.* 1996). These studies suggested that the presence of this sequence within *E. coil*-derived plasmids may

also have an effect. Indeed, vectors with two copies of the sequence AACGTT were more immunogenic in mice, as measured by antibody titers and cell-mediated immune responses, than were similar vectors not containing this sequence (Sato *et al.* 1996). In addition, studies have shown that co-injection of mice with NP protein and a plasmid DNA not containing a gene insert changed the antibody isotype profile induced against NP to a predominance of IgG2a from a predominance of IgG1 when mice were vaccinated with NP protein alone (Donnelly *et al.* 1992). Of related interest were the observations (Gilkeson *et al.* 1995) that showed that vaccination of spontaneously autoimmune *NZB/NZW* mice with bacterial DNA hastened the onset of anti-DNA antibodies, but, surprisingly, protected mice from progressing to autoimmune disease (Gilkeson *et al.* 1996). In these studies, potential immunomodulating effects of the DNA may have played a role in the attenuation of disease. These results open the interesting possibility that engineering vectors to include copies of these stimulatory nucleotide sequences may have profound effects on the quality and quantity of immune responses against encoded antigens.

11.1.4. D8. Enhancing Delivery into the Major Histocompatibility Complex Class I Pathway. CTL responses can be enhanced by engineering the antigen to target specific cellular compartments. An example for this engineering is the use of N-terminal ubiquitination signals, which target the protein to proteosomes, leading to rapid cytoplasmic degradation and presentation via the MHC class-I pathway. In this regard, it was demonstrated that a DNA vaccine encoding β-gal that was fused with ubiquitin was more effective at inducing CTL responses than was a plasmid encoding β-gal alone. The latter construct was also less efficient at inducing antibody responses, suggesting that the transfected gene product was rapidly degraded intracellularly and that processing precluded the release of native polypeptides or proteins for efficient antibody production (Wu *et al.* 1997). These results are in agreement with studies in other model systems targeting HIV Nef (Tobery *et al.* 1997) and LCMV nucleoprotein (Rodriguez *et al.* 1997).

Another approach is to design vectors that use the E3 leader sequence from adenovirus, which facilitates transport of antigens directly into the endoplasmic reticulum for binding to MHC class-I molecules, bypassing the need for the TAP transporter. The addition of the E3 leader sequence appeared to improve CTL

responses for certain antigens (Ciernik *et al.* 1996; Iwasaki *et al.* 1999) but did not improve CTL in other model systems (Ciernik *et al.* 1996). These data suggest that endoplasmic reticulum-targeting of T-cell epitope DNA vaccines may not enhance the immune response for all antigens.

11.1.4. D9. Effects of Manipulating Heterologous Genes on the Immune Response. Optimizing codon usage for eukaryotic cells can also enhance expression of antigens. Codon bias has been observed in several species, and the use of selective codons in a particular gene correlates with efficiency of gene expression (Lewin 1994). This correlation was shown by using a plasmid expressing listeriolysin O, in which codons frequently used in murine genes were substituted for the native codons for the encoded antigen. This substitution led to enhanced CTL and protective immunity (Uchijima *et al.* 1998). Similar results were noted in mice, by using the HIV-1 gp120 sequence (Andre *et al.* 1998) or gp160 sequence (Vinner *et al.* 1999). A plasmid may also be engineered so that the encoded protein is either secreted or localized to the interior of the cell. Several studies show that the type and magnitude of the immune response depend on whether an antigen is secreted, bound on the surface of the cell, or retained within the cell. For example, secreted proteins induced higher IgG titers than the same antigen localized either on the cell membrane or within the cell (Boyle *et al.* 1997; Lewis *et al.* 1997; Inchauspe *et al.* 1997). It is unclear from these studies how DNA immunization induces antibody production against intracellular, non-cytopathic proteins, because B cells require free or membrane-bound linear determinants or conformational epitopes to initiate the process of clonal expansion for efficient antibody production. These concepts suggest that a non-secreted intracellular antigen would not elicit antibody production (Akbari *et al.* 1999). The evidence that the nature of the antigen used (secreted vs intracellular) can preferentially bias T-helper responses is less clear. In two separate studies, it was demonstrated that secreted antigens induced a higher IgG1:IgG2a ratio (suggesting a Th2 bias) than did antigens that remained cell associated (membrane anchored or cytosolic); however, these studies analyzed antibody subtypes rather than directly measuring cytokines and thus provide only a surrogate for T-helper responses (Haddad *et al.* 1997; Lewis *et al.* 1997).

In a separate study, plasmid DNA expressing either secreted or intracellular antigen induced comparable levels of antigen-specific IFN-γ on in vitro stimulation (Inchauspe *et al.* 1997). Taken together, these data suggest that cellular localization of the antigen after DNA immunization may play a role in modulating immune responses, although this role may depend on the nature of the antigen and model system used (Rice *et al.* 1999).

CHAPTER 3

DNA CHIMERAS AND RECOMBINANT PROTEINS

INTRODUCTION

A novel approach to the development of needed vaccines uses deoxyribonucleic acid (DNA) for immunization. DNA represents the genetic blueprint for life. When DNA is used for immunization, the DNA in the plasmid form provides the code for the vaccinating protein, thus, representing the simplest embodiment of a vaccine that provides genes encoding the antigen (Ag) rather than consisting of the Ag itself. DNA vaccines are administered in saline using hypodermic needles or by propelling DNA-coated gold beads into the skin using gene guns. The actual production of the immunizing protein, therefore, takes place in the DNA inoculated host, initiating both humoral and cellular immunity.

The development of DNA vaccines grew from efforts to generate MHC class I-restricted CTL responses by capitalizing on the understanding of different intracellular Ag-processing pathways. It had become understood that proteins synthesized in somatic cells could generate peptides that would associate with MHC class I molecules for presentation to $CD8^+$ lymphocytes with their subsequent activation. Thus, because the focus of vaccine development expanded to include cellular responses as well as Abs, means were sought to introduce proteins into both the MHC class I & II processing pathways.

MHC II-directed antigen activation of $CD4^+$ T-cells is vital to the function of genetic vaccines as demonstrated in studies with MHC II knockout (Chan *et al.* 2001) or $CD4^+$ depleted mice (Maecker *et al.* 1998). This includes $CD8^+$ responses, which require $CD4^+$ T-helper cells for secondary expansion and the development of memory (Janssen *et al.* 2003). In general, antibody class switching, clonal expansion of antigen specific B cells, T cell expansion and memory cell formation, and many other functions of the immune response require the co-stimulatory signals and cytokines released by antigen-activated $CD4^+$ T cells (Kaech *et al.* 2002). Efficient priming of $CD4^+$ T-cells by DNA vaccination requires sufficient levels of antigen expression in transfected cells and delivery of the protein antigen to the MHC II antigen-processing pathway. This may be facilitated by direct transfection of dendritic cells, a key event in the function of DNA vaccines (Guermonprez *et al.* 2002). However, although there is evidence for activation of $CD4^+$ T cells by DNA-

encoded proteins (Moreno *et al.* 1991; Robinson *et al.* 2002), epitopes of endogenous proteins expressed in the cytoplasm of transfected cells are commonly presented by MHC class I molecules to cytotoxic $CD8^+$ T-cells. Antigens presented by MHC II molecules to helper $CD4^+$ T-cells are derived mainly from extracellular "foreign" proteins taken into dendritic cells by endocytic receptors (Robinson *et al.* 2002; Théry *et al.* 2001).

With a goal of enhancing endogenous antigen trafficking to the cellular MHC II compartment of antigen-presenting cells, we employed the use of a genetic vaccine encoding the antigens as a chimera containing the lysosomal targeting sequences of the lysosome-associated membrane protein 1 (LAMP 1). Our rationale was that the delivery of antigen to the cellular site of MHC II processing and binding of antigen epitopes could result in an enhanced immune response through greater antigen-specific activation of $CD4^+$ T-cells. This would in turn be revealed by the spectrum of B- and T-cell responses represented by initial clonal outburst, polarization of T-helper cell responses (TH1/TH2), antibody isotype switch and B- and T-cell memory responses. Also, TPA leader sequence (Tissue plasminogen activator) has been shown to facilitate the secretion of the encoded antigen (Liang *et al.* 2005). Therefore, its inclusion in DNA construct may help facilitate secretion of vaccine-encoded antigen that could well be taken up as an exogenous antigen by phagocytosis or endocytosis by APC and directly targeted to endosomal pathway.

In addition to efficient antigen targeting and presentation of antigen via the MHC II pathway, effective $CD8^+$ T cell responses are dependent upon efficient processing through MHC I pathway alongwith the help provided by $CD4^+$ T cells (Castiglioni *et al.* 2005). For that we took advantage of the fact that carboxy terminus of UQ (Ubiquitin) is a substrate for a site-specific protease (Holowaty *et al.* 2003). Rationale was, ubiquitination should target the protein for rapid cytoplasmic degradation by the proteasome and increase the availability of the antigenic peptides for presentation through the MHC I pathway (Rock *et al.* 1994). Subsequently, immune recognition should be reflected in the magnitude of Ag-specific $CD8^+$ T cell-mediated immune responses generated.

A point of great interest is to note that ATR resides in the glycerolipidic i.e. non-raft regions of the plasma membrane. However, upon binding and heptamerization of PA, the toxin-receptor complex associates with lipid-raft like

domains and undergoes rapid endocytosis via clathrin-dependent pathway (Abrami *et al.* 2003). This process subsequently leads to the translocation of the enzymatic subunits into the cytosol through the protective antigen pore. This kind of behavior parallels B-cell receptor which also undergoes ligand-dependent clustering and raft association (Cheng *et al.* 1999), and is subsequently internalized via clathrin dependent mechanism (Stoddart *et al.* 2002).

The observation that ATR couples raft translocation and mediates endocytosis of EF/LF along with the oligomerization of PA made us hypothesize that a DNA chimera encoding protease-cleaved fragment of PA (PA63) attached to a C-terminal glycosylphosphatidyl inositol (GPI) anchor sequence might ensure lateral association of the cell-surface GPI-anchored PA with liquid-ordered, cholesterol- and sphingolipid-rich domains or rafts. Biochemical, morphological and functional approaches have been able to trace that GPI-anchored protein rich rafts are transported down to the endocytic pathway to reducing late endosomes in mammalian cells (Fivaz *et al.* 2002).

Apart from that, GPI-anchoring has been attributed to be a positive signal for internalization into rab5-independent (rab5, a small GTPase located in early endosomes) tubular-vesicular endosomes also responsible for fluid-phase uptake (Sabharanjak *et al.* 2002). Therefore, adoption of such an approach opens up a possibility that GPI anchored PA will undergo its natural cycle upon endocytosis that might improve DNA vaccine potency. As a matter of fact, it is also well documented that CD1 molecules (MHC-I like glycoproteins) survey the endocytic pathway to intersect and bind lipid antigens (Barral *et al.* 2007). Both human and muroid CD1d molecules (Group 2 CD1 molecules) have been shown to bind to GPI-anchored proteins and present them to a variety of NKT cells that function against infection with pathogens (Schofield *et al.* 1999). Group 2 CD1 (CD1d) molecules have also been shown to control humoral immunity against parasites (Hansen *et al.* 2003), elicit cell-mediated immune responses against tumors (Cui *et al.* 1997), and induce secretion of large amounts of both inflammatory IFN-γ and immunoregulatory interleukin (IL)-4 and IL-10 cytokines (Yoshimoto *et al.* 1994; Wilson *et al.* 1998). Thus, GPI-anchoring of PA might as well aid its presentation in context with CD1 molecules that have the advantage of limited allelic polymorphism

yet specialized to bind and present a large repertoire of lipids and glycolipids to T cells.

Therefore, with these observations in mind, we designed the present study and cloned 63kDa fragment of protective antigen (PA) of *Bacillus anthracis* in DNA chimeras bearing a set of signal sequences. Results indicated that the PA63 protein along with the targeting signals was being properly recognized by the mammalian cells. The targeting signals namely, LAMP1 (Lysosome Associated Membrane Protein 1), TPA (Tissue Plasminogen activator), Glycosyl phosphatidyl inositol (GPI) and Ubiquitin encoded various forms of PA viz. lysosomal, secreted, membrane-anchored and cytosolic, respectively. Regardless of the signal sequence attached, all the constructs showed comparable expression levels. In addition, we cloned PA63 gene fragment in pQE30 expression plasmid (T7 expression system, Qiagen). This construct expressed recombinant PA63 (rPA63) protein to homogeneity. Purified rPA63 mediated its function as the receptor binding moiety of anthrax toxin complex in a manner similar to that displayed by full-length rPA83 protein

MATERIALS AND METHODS

BACTERIAL STRAINS

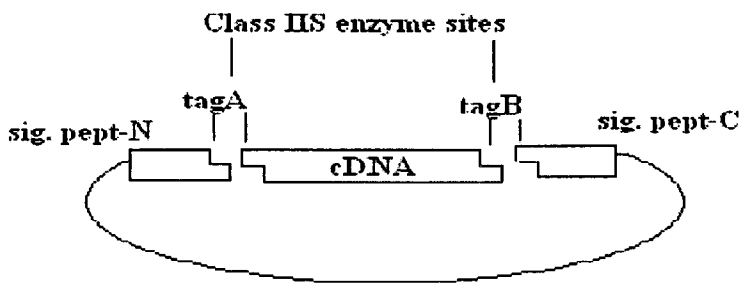
E. coli strain DH5α was used in all cloning experiments for generation of new constructs and for plasmid propagation. The *E. coli* strain used in this study for the expression of the cloned genes was *E. coli* M15. Both the strains were obtained from Invitrogen Corp (USA).

DNA VACCINE VECTORS

DNA vaccine plasmids utilized were procured from Nature Technology Corp. (NE, USA). These plasmids have been created by single step assembly of six precloned modules using gene self assembly (GENSA) technology.

GENSA uses class IIS restriction enzymes to generate unique, non-palindromic overhanging termini that can ligate to only one other terminus in a complex mixture, thus assuring that each fragment ligates in the correct orientation to its correct partner.

A.



B.

5'-GCGCTTC N ^GGG^NNNNNNNNNNN-3'
SapI - tag - primer

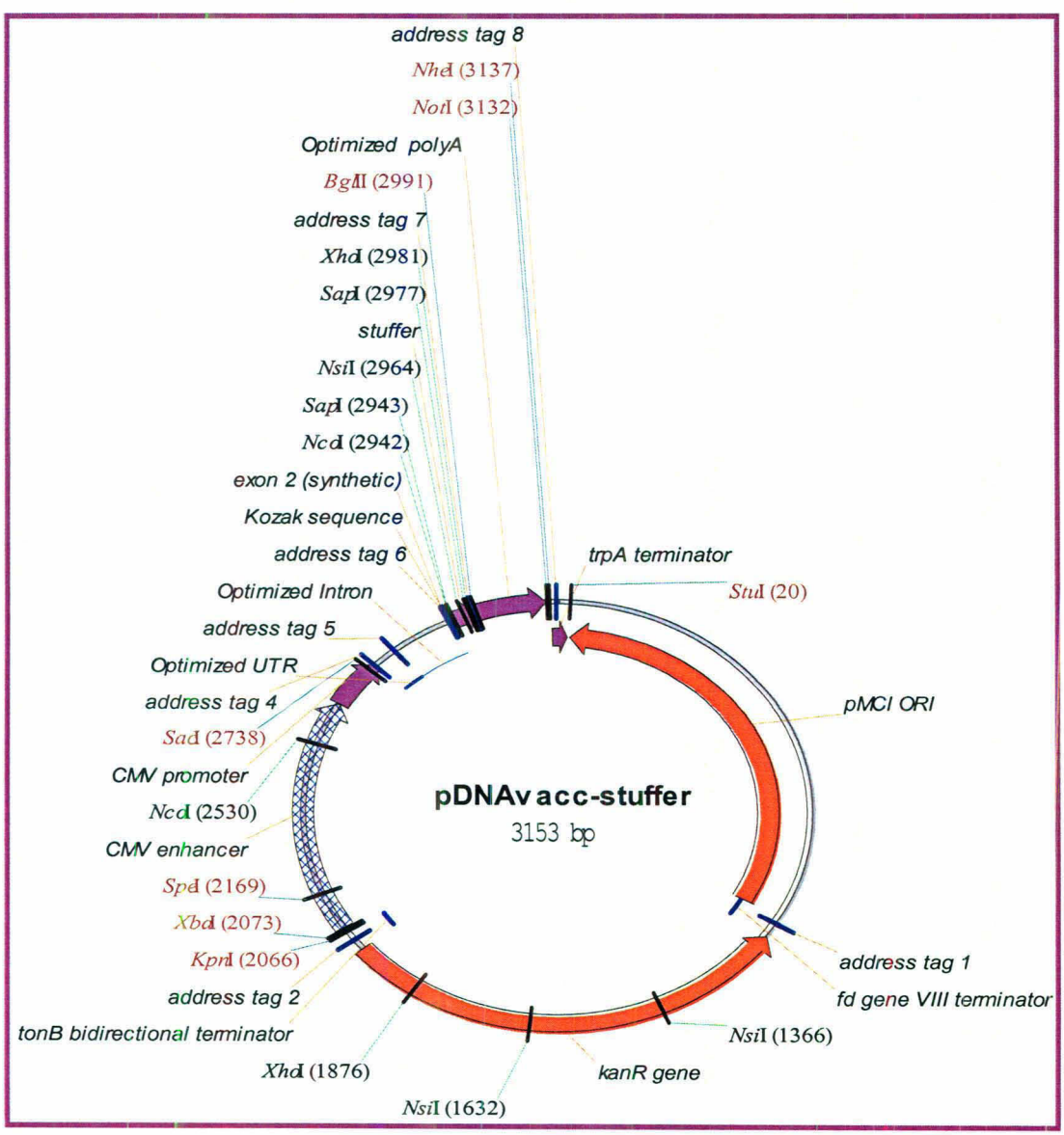
The (GENSA) modules consist of:

- High copy number pUC prokaryotic replication origin;
- Prokaryotic selectable marker gene (kanamycin; kanR);
- Eukaryotic enhancer-promoter (CMV)

pDNAVACC vector family:

- Synthetic eukaryotic untranslated leader-intron-translational initiation sequence (Kozak sequence) cassette derived from rabbit β-globin leader and intron
- High throughput seamless cloning site targeting gene leader cassette (basis for difference between pDNAVACC1-7).
- Synthetic eukaryotic transcriptional terminator based on rabbit β-globin.

BASIC MAP OF pDNAVACC VECTOR FAMILY



RESTRICTION ENDONUCLEASES AND DNA MODIFYING ENZYMES

Restriction endonucleases, T4 DNA ligase used in all cloning experiments were procured from MBI, Fermentas.

KITS

Commercially available kits for preparation of plasmid DNA and for elution of DNA from agrose gel were obtained from Qiagen. Cell transfection kit was obtained from Invitrogen Corp (USA).

MARKERS AND STANDARDS

1kB DNA ladder, protein ladder, and pre-stained high molecular protein marker for SDS-PAGE gels were procured from MBI, Fermentas.

CELL-CULTURE REAGENTS AND CELL-LINES

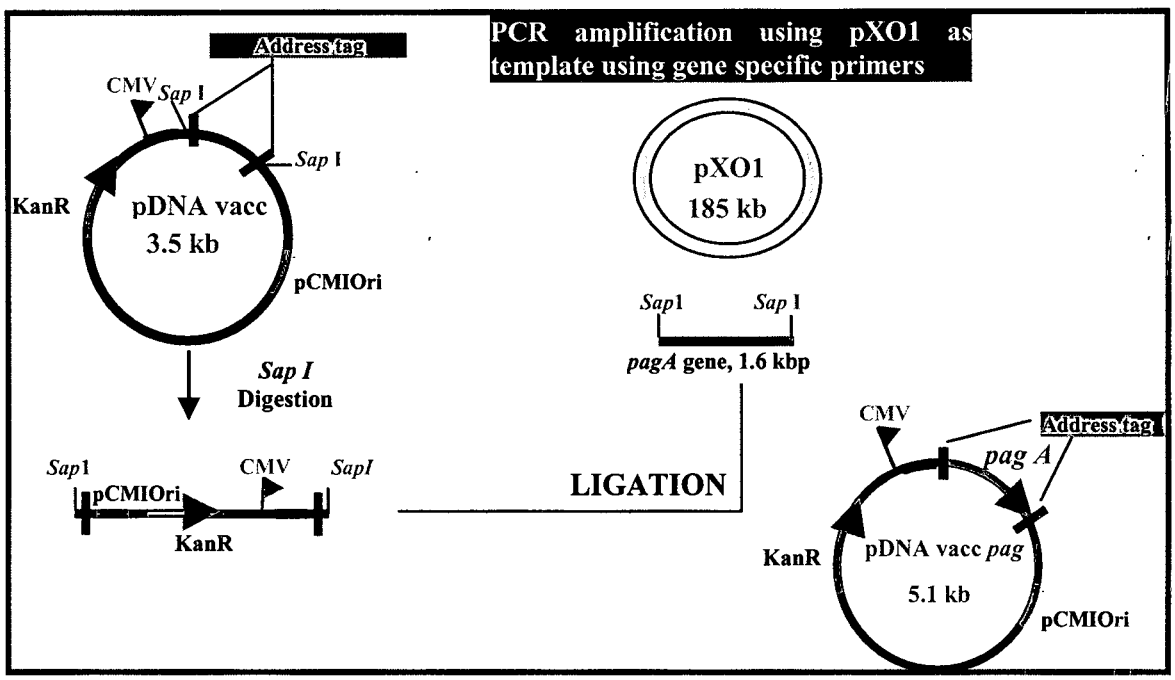
RPMI 1640 medium was obtained from Sigma. Fetal bovine serum was obtained from Biological industries. J774A.1 mouse macrophage-like cell line was obtained from ATCC, USA. Cell culture plasticware was obtained from Corning–Costar, USA.

PRIMERS

The oligonucleotides used for cloning experiments were obtained from Sigma-Aldrich.

PRIMER SEQUENCE	N or C-TERMINAL LINKER	ADDRESS TAG
Forward primer: (5'-GCGCAT GCTCTTCCATGCCTACGGTTCCAGACC G -3')	ATG (N-terminal)	All vectors
Reverse primer 1 (5'-GCAGAAGCTCTTCGTTATCCTATCTCATAGCCTTTTTTA-3')	TTA (C-terminal)	Ubiquitin, Native, Secreted
Reverse primer 2 (5'- GCAGAAGCTCTTCGGCCTCCTATCTCATAGCCT TTTTTA G-3')	GCC (C-terminal)	Endosomal Secreted-endosomal

STRATEGY USED CLONING PA GENE IN TARGETED VECTORS



Intron ▼ Kozak ← SapI SapI →
1 CAGGCCGCCACCATGGGAAGAGCGTTCCATGCATCCTAGCTCTTCGTAAC

XhoI BglII
51 TCGAGCCGCA GATCT
ATG TAA = address tags

The PA gene was amplified using pXO1 plasmid from *Bacillus anthracis* (Sterne strain) as template and cloned in pDNAVACC plasmids using the following strategy:

1. Forward Primer A, corresponding to nucleotides 604-619 and spanning a unique *Sap*I site was used either with reverse 1 or 2 (with a stop codon or a GCC linker) spanning nucleotides 2270-2292 of PA, for a round of PCR amplification using pXO1 as template.
2. The amplified products obtained from both the reaction mix (Reverse primer 1 and Reverse primer 2) after the first round of amplification, were then digested with the enzyme *Sap*I.
3. pDNA vacc plasmids were also digested with *Sap*I to remove the stuffer sequence between the two *Sap*I sites and generate backbone for ligation.

The digested PCR product was ligated with the vector backbone to generate recombinant construct containing the PA gene.

PCR AMPLIFICATION OF PROTECTIVE ANTIGEN GENE

Protease cleaved fragment of the structural gene for protective antigen (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid. The primers utilized for amplification introduced *Sap*I sites at the 5' and 3' ends of PCR product, respectively.

The reaction mix consisted of 50ng of template DNA, 0.2mM of each dNTP (MBI, Fermentas), 1 μM of both the primers, 10 μl of Taq DNA polymerase buffer (10X) and 1 unit of Taq DNA polymerase (Bangalore genei). The PCR conditions were:

PCR cycle:

Initial denaturation	94°C	5 min	
Thermo cycle	94 °C	45 sec	30 cycles
	68°C (Set I)*	30 sec	
	64°C (Set II)*	30 sec	
	72°C	2 min	
Final extension	72 °C	7 min	

*Annealing at 68°C (for Reverse Primer 1 with TAA stop codon) and 64°C (for Reverse primer 2 with GCC glycine linker) for 30 seconds.

CLONING OF PA63 GENE FRAGMENT IN pQE30 VECTOR

Protease cleaved fragment of the structural gene for protective antigen (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid. The primers utilized for amplification introduced *BamH*I and *Kpn*I sites at the 5' and 3' ends of PCR product, respectively. PCR was performed in 100µl reaction using DNA thermal cycler (Perkin Elmer) in 0.2 ml thin walled tubes. The reaction mix consisted of 50ng of template DNA, 0.2mM of each dNTP (MBI, Fermentas), 1 µM of both the primers, 10 µl of Taq DNA polymerase buffer (10X) and 1 unit of Taq DNA polymerase (Bangalore genei).

The PCR conditions were:

Initial denaturation	94°C	5 min	
Thermo cycle	94 °C	45 sec	30 cycles
	50°C	30 sec	
	72°C	2 min	
Final extension	72 °C	7 min	

Primer Sequences:

1) **Forward Primer**: 5'-CGG GGT ACC ATG CCT ACG GTT CCA GAC CG-3'

2) **Reverse Primer:** 5'-CGC GGA TCC GAT CCT ATC TCA TAG CCT TTT TTA G-3'

EXTRACTION OF DNA FROM AGAROSE GELS

The PCR reaction mix was fractionated by agarose gel electrophoresis. The fragments of the right size were excised from the agarose gel and DNA was eluted from the gel slice using gel extraction kit (Qiagen) as prescribed by the manufacturer:

1. The gel piece containing the DNA fragment of interest were chopped into fine pieces and transferred to a 1.5ml microfuge tube. To this, 4-5 gel volumes of solubilization buffer Q XI was added and incubated at 55°C for 10-15 min.

2. 100 µl of isopropanol and 10 µl of sodium acetate (pH5.2) were added to the microfuge tube.

3. The sample was loaded onto Qiaquick gel extraction spin column and centrifuged at 14,000 rpm for 1 min.

4. The column was washed with 750 µl of wash buffer PE followed by a spin at 14,000 rpm for 1 min. To remove the residual PE, the column was again spun for 1 min.

5. The column was air dried by keeping in 37°C incubator for 10 min.

6. The column was kept in a fresh microfuge tube and the DNA was eluted in 50 µl autoclaved Milli-Q water by spinning the column at 14,000 rpm for 5 min.

LIGATION

The digested fragments were resolved on 1% agarose gel and were extracted from the gel as described earlier. The ligation reaction was set up in 10 µl reaction volume with backbone DNA (10ng), digested PCR-product (30ng), 1.0 µl of ligation buffer (10x), 1 unit of T4 DNA ligase. The ligation reaction mixture was incubated overnight at 4°C.

COMPETENT CELL PREPARATION

The procedure used for the preparation of competent cells was a slight modification (Cohen *et al.* 1972). It was frequently used to prepare competent bacteria of *E. coli* strain, DH5α and M15, which yielded $5x10^6$ to $2x10^7$ transformed colonies per microgram of supercoiled plasmid.

1. The host cell culture was streaked on a LB plate from the frozen glycerol stock at -80°C. A single colony was inoculated into 3 ml LB. One ml of the overnight grown culture was further inoculated into 100 ml LB and allowed to grow for 2-3 hrs until A_{600} reached 0.4-0.5. The culture was chilled on ice, transferred to ice cold 50 ml polypropylene tubes and centrifuged at 4000 rpm for 10 min in a Sorvall SS34 rotor.

2. The supernatant was decanted and the pellet was re-suspended gently in 10 ml of ice cold 0.1 M $CaCl_2$ and incubated on ice for 10 min.

3. The cells were then centrifuged at 4000 rpm in Sorvall SS34 rotor. The pellet was re-suspended in 3 ml of ice cold 0.1 M $CaCl_2$. Chilled glycerol was added to the cells to a final concentration of 20%.

4. About 200 µl aliquots were taken for checking viability, contamination and efficiency of transformation. The rest of the suspension was kept at 4°C for 12-24 hours to increase the efficiency of transformation and stored in aliquots of 200µl at – 80°C.

TRANSFORMATION OF DNA INTO COMPETENT CELLS

Competent *E.coli* DH5α cells were transformed with the ligation mixtures of the DNA vaccine plasmids, following regular molecular biology procedures (Sambrook *et al.* 1989). Same procedure was used to transform *E.coli* M15 cells with the pQE30 clone containing PA63 gene.

1. The competent cells aliquot of 200 µl was thawed over ice and DNA was added, mixed by tapping and kept on ice for 30 mins.

2. The cells were subjected to heat shock at 42°C for 90 seconds in a water bath and were immediately transferred to an ice bath. The cells were allowed to chill for 1-2 mins.

3. 800 µl of LB was added to the cells.

4. Cells were then incubated at 37°C for 1hr on a shaker.

5. The cells were pelleted and then re-suspended in 100 µl of LB. The re-suspended cells were plated on LB-agar plates containing kanamycin and ampicillin (for pQE30 vector clone) and only on kanamycin (for pDNAVACC clones). Antibiotic concentrations used: 100 µg/ml ampicillin and 50 µg/ml kanamycin.

6. The plates were incubated at 37°C for 16 hours.

SCREENING OF THE TRANSFORMANTS

The transformants were screened for the presence of insert by two methods:

1. Rapid colony prep to check the presence of PA gene in the putative colonies based on gel shift assay.

2. Minipreparations of plasmid DNA followed by restriction analysis of the recombinant constructs.

1. RAPID COLONY PREP

All the colonies obtained after ligation reaction were screened using a rapid Colony prep. Briefly, solution I [50mM Glucose, 25mM Tris.HCl (pH 8.0), and 10mM EDTA] and solution II [SDS (1%) + NaOH (0.2N))] were mixed in the ratio of 1:1 along with 1X loading dye. In an eppendorf, 40 µl of this solution was taken and a little amount of scrapped colony was picked from the master plate and emulsified in this solution. The resulting cell lysate was loaded directly in the wells of an agarose gel and run at 150V. The colonies that harbor a clone show a shift as compared to the control vector.

2. MINIPREPARATION OF PLASMID DNA

The method used for minipreparations of plasmid DNA is a modification of the methods described by Birnboim and Doly (1979) and Ish-Harowicz and Burke (1981).

1. A single transformed colony was inoculated in 3ml of LB medium containing ampicillin and shaken overnight at 37°C. The culture was pelleted in a microfuge tube at 12,000*xg* for 30 seconds.

2. The media was discarded and the pellet was resuspended in 150µl of ice-cold solution I (50Mm glucose, 25mM Tris-Cl pH 8.0 and 10mM EDTA pH 8.0) by vigorous vortexing.

3. To the tube, 200 µl of freshly prepared solution II (0.2N NaOH, 1% SDS) was added. The contents of the tube were gently mixed by inverting the tube four to five times and incubated at room temperature for five minutes.

4. After 5 minutes, 150 µl of ice-cold solution III (5M potassium acetate, glacial acetic acid and water) was added. The contents of the tube were mixed and the tube was incubated on ice for 15 minutes.

5. The mixture was centrifuged at 12,000*xg* for 10 minutes. The supernatant was transferred to a fresh tube and 0.7 volume of isopropanol was added to it.

6. The DNA was precipitated by centrifugation at 13,000*xg* for 20 minutes at room temperature. The pellet was washed with 20% ethanol and air-dried. The air-dried pellet was resuspended in 20 µl of autoclaved Milli-Q water.

RESTRICTION ANALYSIS OF THE RECOMBINANT CONSTRUCTS

400 ng of plasmid DNA was sequentially digested with 0.5 unit each of *EcoR* I and *Hind* III enzymes (for the DNA vaccine chimeras) and with *Bam* HI and *Kpn* I (for PA63 clone in pQE30 vector) in a 20 µl reaction mix containing 4µl of the 10x-R^+ buffer for the restriction endonucleases. The digestion mixtures were incubated at

37°C for two hours before running on 1% agarose gel along with a standard marker, to check for the presence of insert of the right size. Clones selected after restriction analysis were then sequenced to confirm that the desired gene has been cloned (For chromatograms see *Appendix*). Finally the nucleotide sequence of the DNA chimeras bearing the address tags was submitted to GENEBANK.

EXPRESSION OF RECOMBINANT PA63

E. coli M15 transformants carrying the plasmid pQE30 with the cloned PA63 gene, were grown at 37°C in 1litre of LB medium containing 100 μg/ml ampicillin and 50 μg/ml of kanamycin at 250 rpm. When A_{600} reached 0.8-1.0, IPTG was added to a final concentration of 1.0 mM. After 3 hours of induction, at 37°C, the cells were harvested by centrifugation at 4,000 *xg* for 10 minutes at 4°C. The cell pellet was stored at -80°C till further use.

PURIFICATION OF RECOMBINANT PROTECTIVE ANTIGEN

As PA was mainly localized in the inclusion bodies, the protein was purified under denaturing conditions. The pellet from 2 liters of culture was resuspended in 100 ml of buffer containing 8 M Urea, 0.1 M Na-phosphate, pH 7.8, and 300 mM NaCl. Cells were stirred at room temperature for 1 h. Lysate was centrifuged at 10,000*g* for 30 min at room temperature. The supernatant was mixed with 8 ml of 50% slurry of Ni-NTA resin and allowed to stir at room temperature for 45 min, and then the resin was loaded carefully into a 1.6-cm diameter column. The column was washed with 10-column vol. of buffer containing 8 M urea, 0.1 M Na-phosphate, pH 7.8, and 300 mM NaCl. The resin was then washed with a gradient of 8 to 0-M urea in buffer containing 0.1 M Na-phosphate, pH 7.8, and 300 mM NaCl to facilitate the slow removal of the urea. The resin was then washed with an additional volume of 50ml chilled buffer containing 0.1 M Na-phosphate, pH 7.8, and 300 mM NaCl, to remove the traces of urea. An additional purification step was performed to remove endotoxin contaminations (lipopolysaccharide) using a procedure described previously (Franken *et al.* 2000). The recombinant protein was eluted by pre-chilled elution buffer containing 250 mM Imidazole in 0.1 M Na-phosphate, pH 7.0, and 10% glycerol. The fractions were analyzed on SDS–PAGE and those containing the

protein were pooled and dialyzed against $T_{10}E_5$ (Tris 10 mM and EDTA 5 mM, pH 8.0) buffer at 4°C. The dialyzed sample was loaded onto the Mono-Q column. The protein was eluted with a gradient of 0 to 1 M NaCl in $T_{10}E_5$. The rPA was dialyzed against 10 mM HEPES overnight and stored in aliquots at -80°C.

POLYACRYLAMIDE GEL ELECTROPHORESIS

A 12% separating gel was utilized for the electrophoretic analysis of proteins. For stacking of proteins 5% gel was used. Protein samples for SDS-PAGE were prepared by the addition of the 6X loading buffer to the final concentration of 1X, boiled for 5 minutes followed by centrifugation at 12,000g for 5 minutes at room temperature. Proteins were analysed by SDS-PAGE (Laemmli 1970) at a constant voltage of 100V in a Bio-Rad mini gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The resolved proteins were visualized by staining the gels with Coomassie Brilliant Blue R-250 for 30 minutes followed by destaining the gels to remove excess stain.

WESTERN BLOT ANALYSIS

Protein samples resolved on SDS-polyacrylamide gels were transferred to nitrocellulose membrane at a constant voltage of 30V overnight or 100V for an hour in the Bio-Rad mini trans-blot cell. Blocking was carried out by using 3% BSA in TTBS for 1 hour. For probing the blot with antibody, the membrane was incubated for 45 minutes with rabbit anti-PA antibody, diluted in PBS (pH 7.2) containing 0.5% BSA. Blot was washed with three changes of TTBS and then probed with donkey anti-rabbit IgG-alkaline phosphatase conjugated secondary antibody (Amersham biosciences) for 45 minutes. The membrane was washed three more times with TTBS and was developed by addition of NBT/BCIP (Sigma Aldrich) as substrate.

TRANSFECTION

J774.A1 mouse MΦ-like cells were seeded at a concentration of 2-3 × 10^7 cells into a 75-cm^2 flask (Corning costar) until the cells reached approximately 50–70% confluence. Plasmid DNA transfection was performed with LipofectAMINE 2000 (Invitrogen) reagent, as specified by the manufacturer.

SUBCELLULAR FRACTIONATION AND WESTERN BLOT ANALYSIS

Transfected J774A.1 cells were washed in ice-cold Hanks buffered salt solution (HBSS), gently scraped into fresh HBSS (10 ml) and centrifuged at 145g for min. The pellet was washed in homogenization buffer, pH 7.0 (4°C, 0.25M sucrose; 20mM HEPES; 0.5mM EDTA), re-centrifuged as above and finally re-suspended in fresh homogenization buffer (1.5 ml). Whole cells were ruptured as described previously (Balch *et al.* 1984) at a concentration of 1.5-2.0 × 10^7 cells/ml. The ruptured cells were centrifuged at 800g for 10 min at 4°C to remove unbroken cells and nuclei. Ficoll (Pharmacia) and Nycodenz (Nycomed) density gradients were prepared as described earlier (Brown *et al.* 2000). The cell supernatant was layered on the top of the gradient and the sealed tube was centrifuged in Beckman VTi 65.2 rotor at 240,000g and 4°C for 90 min, using slow acceleration and deceleration programs. Fractions were collected (26x200µl) from the bottom of the centrifuge tube and stored on ice. The presence of lysosomes in different fractions was determined by analyzing the activity of β-hexosaminidase (Koldovský & Palmieri 1971). The cell membrane fractions were isolated by determining the NADH oxidase activity associated with the cell membranes (Rezwan *et al.* 2007). The J774A.1 culture supernatant proteins were precipitated by ice-cold acetone. For western blot analysis, the total cells were lysed in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) 36 h post transfection. The proteins from the lysosomal fraction, cell-culture supernatant and cell membrane were solubilized in solubilization buffer (60mM TriS-HCl (pH6.8), 1% β-mercaptoethanol, 1% SDS, 10% Glycerol). Finally, the solubilized proteins from the total cell lysate, lysosomes,

cell membrane and cell-culture supernatants were subjected to 12% SDS–polyacrylamide gel electrophoresis under denaturing conditions with 50 mM dithiothreitol (DTT). Proteins from the gel were transferred to a nitrocellulose membrane and probed with rabbit anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG (Amersham biosciences).

RESULTS

Construction of various DNA vaccine chimeras. The structural gene for PA63 was cloned in DNA plasmids bearing the address tags. The colonies after ligation were subjected to rapid colony prep as described in materials and methods section. All the putative clones showed a shift as compared to control vector plasmid *(Figure.1. A-G).*

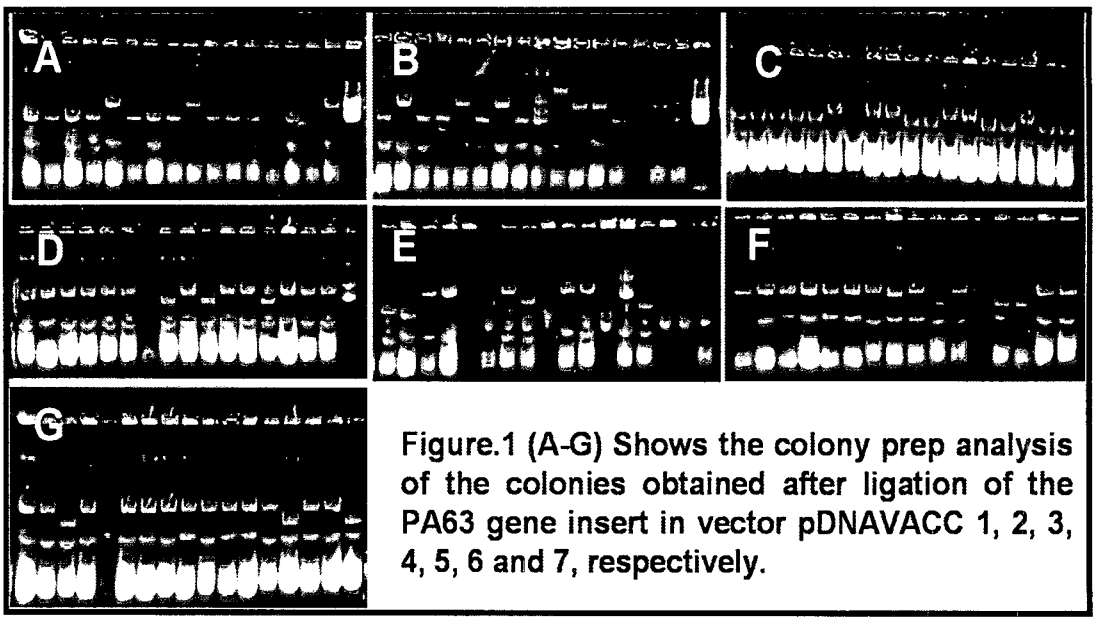


Figure.1 (A-G) Shows the colony prep analysis of the colonies obtained after ligation of the PA63 gene insert in vector pDNAVACC 1, 2, 3, 4, 5, 6 and 7, respectively.

These putative clones were further selected for mini plasmid preparations. Plasmid DNA preparations was sequentially digested with 0.5 unit each of *EcoR* I and *Hind* III enzymes to check for the insert of right size. Digestion with these two restriction endonucleases resulted in two fragments of size 2.45 kb and 2.64 kb which corresponded well with the theoretical as well as the calculated size of fragments/ clone (5.1 kb clone, 3.5 kb vector and 1.6 kb PA63 gene fragment) upon restriction digestion *(Figure. 2).* Finally the nucleotide sequences of clones bearing the address tags was confirmed by sequencing (for sequence chromatograms see *Appendix*) and the sequences were submitted to the Gene Bank, pPA63-Native: (GenBank accession no. EU249810), pTPA-PA63 (GenBank accession no. EU249808), pUQ-PA63 (GenBank accession no. EU249809), pPA63-LAMP1

(GenBank accession no. EU249806), pTPA-PA63-LAMP1 (GenBank accession no. EU249807).

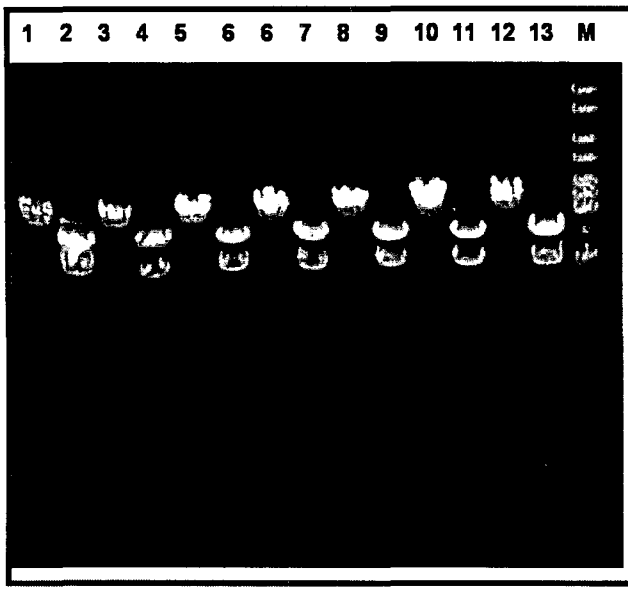


Figure. 2. Restriction analysis of PA63 DNA vaccine chimeras. Lane 1&2. pPA63-GPI clone and vector, Lane 3&4. pUQ-PA63 clone and vector, Lane 5&6. pTPA-PA63 clone and vector, Lane 7 & 8. pPA63-LAMP1 clone and vector, Lane 9 & 10. pTPA-PA63-LAMP1 clone and vector, Lane 11 & 12. pTPA-PA63-GPI clone and vector, respectively, Lane 13. pPA63-Native clone, Lane M-DNA ladder.

Construction of prokaryotic expression vector bearing PA63 gene fragment. The structural gene for protective antigen (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using sequence-primers (see materials and methods for primer sequences) and cloned in pQE30 vector (prPA63).

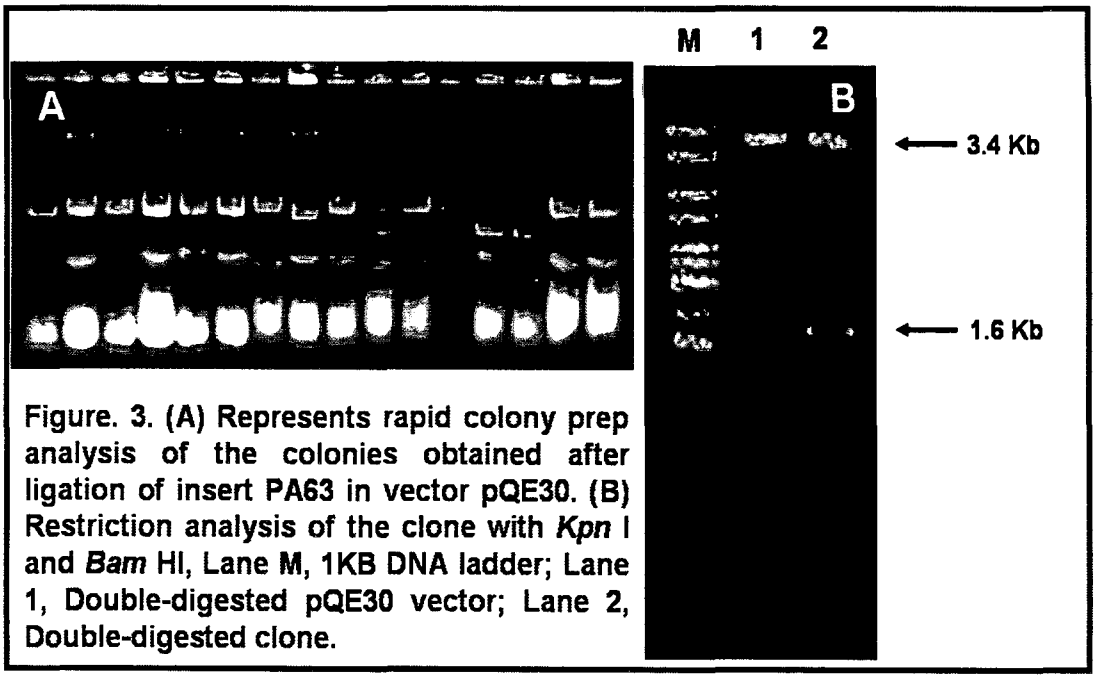


Figure. 3. (A) Represents rapid colony prep analysis of the colonies obtained after ligation of insert PA63 in vector pQE30. (B) Restriction analysis of the clone with *Kpn* I and *Bam* HI, Lane M, 1KB DNA ladder; Lane 1, Double-digested pQE30 vector; Lane 2, Double-digested clone.

The colonies after ligation were subjected to rapid colony prep as described in materials and methods section. All the putative clones showed a shift as compared to control vector plasmid *(Figure.3. A).* The putative clones detected from colony

prep assay were taken forward for mini plasmid preparations. Plasmid DNA preparations was sequentially digested with 0.5 unit each of *Kpn* I and *Bam* HI enzymes to check for the insert of right size. Digestion with these two restriction endonuclease resulted in two fragments of size 3.1 kb and 1.6 kb correspond corresponding to the vector and gene insert, respectively *(Figure. 3B).*

Purification of recombinant protective antigen. The high versatility and high resolving power of ion exchange chromatography made it an ideal choice to purify PA to homogeneity. pI of anthrax protective antigen is 5.5 and the protein is most stable in the pH range of 7.4 to 8.0. The total cell lysate (urea lysate) containing the protein was loaded onto a resource Ni^{2+}-NTA metal affinity column and subjected to purification procedure mentioned in the materials and methods. After an additional lipopolysaccharide removal step, fractions containing protective antigen were eluted from the column. The eluted fractions were analyzed by 12% SDS PAGE and western blotting *(Figure. 4. A & B).* Protective antigen that eluted from the affinity column had >95% purity.

Fig. 4: Purification of *E. coli*-expressed rPA63

(A) SDS-PAGE analysis on a 12% gel

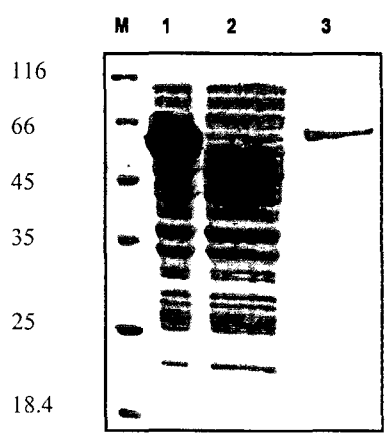


Figure. 4A. SDS-PAGE analysis of total-cell lysate and purified protein.

Lane M - Protein Molecular Weight Marker.

Lane 1 - Induced protein sample.

Lane 3 – Un-induced protein sample.

Lane 4 - Ni^{2+}-NTA Purified RecombinantPA63 protein sample (corresponds to 63 KDa).

(B) Immunoblot analysis with polyclonal rabbit anti-PA antibody.

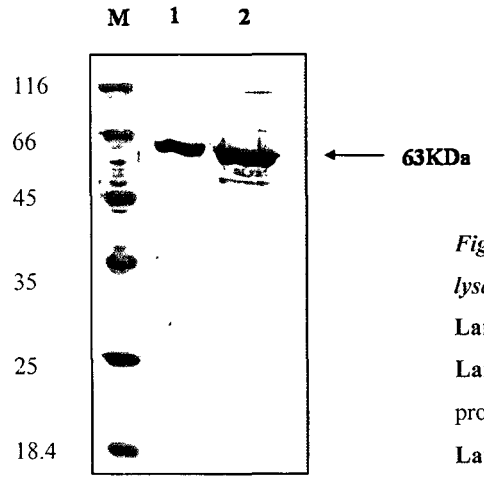


Figure. 4 B. Immunoblot analysis of total-cell lysate and purified protein.
Lane M - Protein Molecular Weight Marker.
Lane 4 - Ni $^{2+}$-NTA Purified RecombinantPA63 protein sample (corresponds to 63 KDa).
Lane 3 – Total-cell lysate.

Cytotoxicity assay. Further the functional and biological activity of the purified recombinant PA63 protein from *E. coli* cultures was carried out. J774A.1 mouse macrophage-like cells are susceptible to lysis by lethal toxin. Cytotoxicity assay performed on these cells *(Table 1)*, by incubating various concentrations of PA with 1.0 μg/ml of LF. Results showed that recombinant PA63 purified from *E. coli* was biologically active and mediated its function as the receptor binding moiety of anthrax toxin complex in a manner similar to that displayed by full-length PA83 protein *(Figure. 5)*.

TABLE 1: Cytotoxicity produced in J774A.1 cells by anthrax lethal toxin

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PA (μg/ml)	Percentage viability [a]	
	PA63	PA83
0.000	100	100
0.001	>98	>98
0.01	~80	~80
0.1	~12	~10
1	0	0

[a] J774A.1 cells were incubated with 1 μg/ml of LF along with the indicated concentrations of PA (purified from either hosts) for 3 hours at 37°C. Cell viability was determined by MTT assay. Untreated cells or cells treated with just one toxin component (PA or LF) had 100% viability. The data values are representative of three different experiments done in triplicates with a standard error of less than 5%.

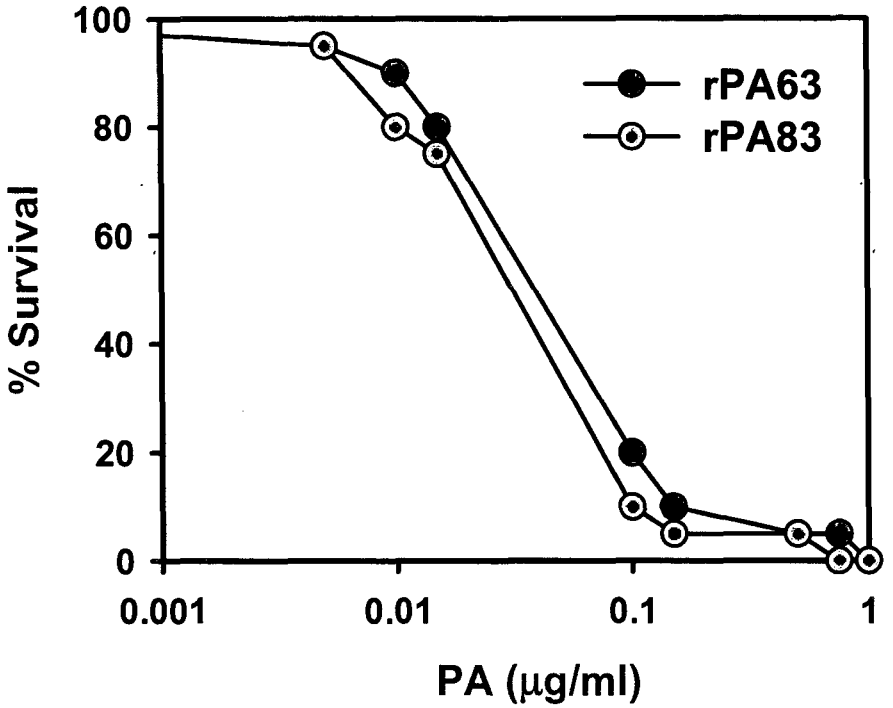


Figure. 5. Biological activity of rPA63 as receptor binding moiety as compared to PA83. The biological activity of rPA63 as receptor binding moiety was established in a standard J774A.1-based cytotoxicity assay and compared with that of rPA83. Results are expressed as % survival of J774A.1 mouse macrophage-like cells.

Sub-cellular fractionation. Lysosomal fractions were isolated by density gradient centrifugation. The 26 fractions were either extracted individually into methanol/ hexane as described in materials and methods. The samples were separated into regions of the gradient containing; free lipids, plasma membrane, endosomes, lysosomes, and the Nycodenz cushion (densities, 1.02, 1.02–1.035, 1.035–1.05, 1.05–1.095, 1.095–1.25 g/ml, respectively. Finally lysosomal fractions were identified by the associated β-hexosaminidase activity *Figure.6.*

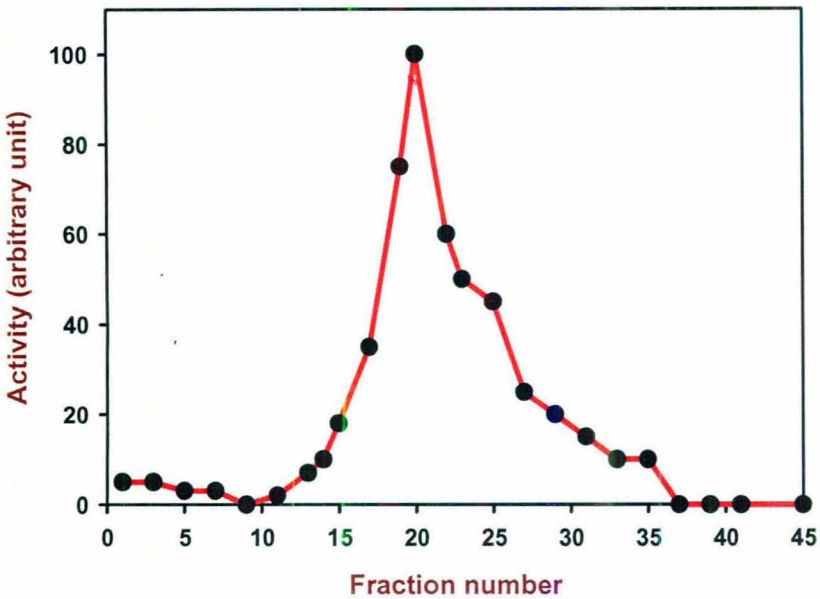


Figure. 6. Detection of β-hexosaminidase activity. β-hexosaminidase activity was measured in the various fractions obtained as a result of sub-cellular fractionation to separate and identify the lysosomal fractions.

Similarly, different fractions were isolated and cell membrane fraction was identified by the associated NADH oxidase activity *(Figure.7)*.

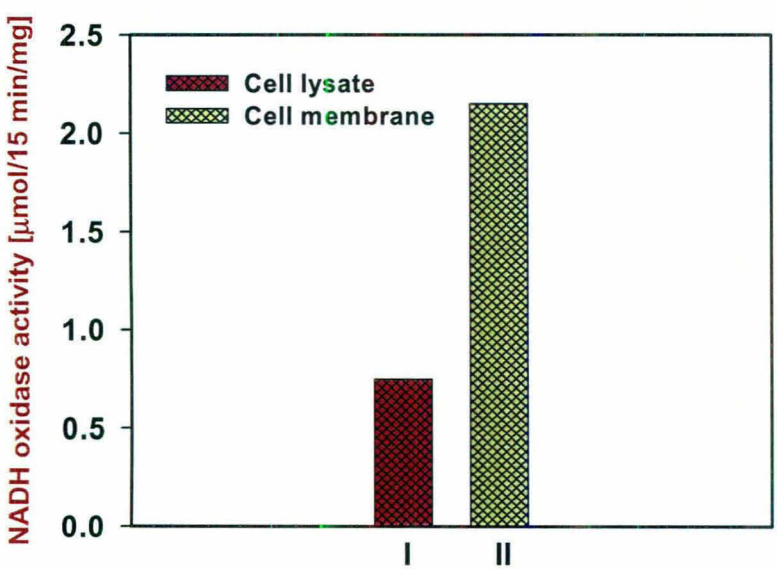


Figure.7. Detection of NADH oxidase activity. NADPH oxidase activity was measured in the various fractions obtained as a result of sub-cellular fractionation to identify and separate the cell membrane fraction.

DNA vaccine chimeras mediated the targeting of PA63 to various sub-cellular locations. Authenticity of the DNA constructs was evaluated by transient transfection in J774A.1 mouse MΦ-like cells followed by subcellular fractionation and immunoblot analysis. *(Figure.8 A & B).* Lysosomal fractions were isolated to check for lysosomal localization of PA63 on account of the attachment of a C-terminal LAMP1 address tag. To detect the membrane-anchored form of PA63 on account of the attachment of GPI-anchor, cell membrane fractions were also isolated. Along with that, cell-culture supernatants were harvested to determine the cellular-secretion of PA63 by TPA leader. SDS-PAGE followed by western blot analysis of the proteins from total cell lysate, lysosome and cell-culture supernatant fractions indicated that PA63 protein along with the targeting signals was being properly recognized by the mammalian cells. As expected, the construct pTPA-PA63 encoded a cell-secreted version of PA63 *(Figure. 8A).* Whereas, the chimera pPA63-LAMP1, expressed a lysosome-associated form of PA63. However, the chimera pTPA-PA63-LAMP1, encoded a lysosome-associated form of PA63 in addition to a cell-secreted version. The construct pUQ-PA63 solely encoded a cell-associated form. Similarly, the DNA chimeras, pGPI-PA and pTPA.GPI-PA, expressed membrane-anchored form of PA *(Figure. 8B).* Along with that the chimera pTPA.GPI-PA also mediated secretion of PA in the culture supernatants.

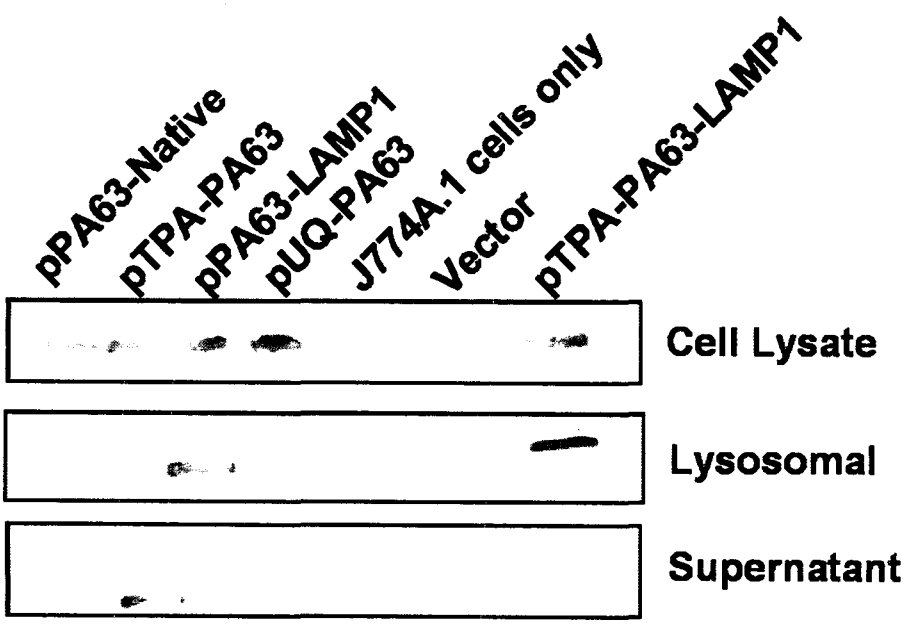


FIGURE.8A.

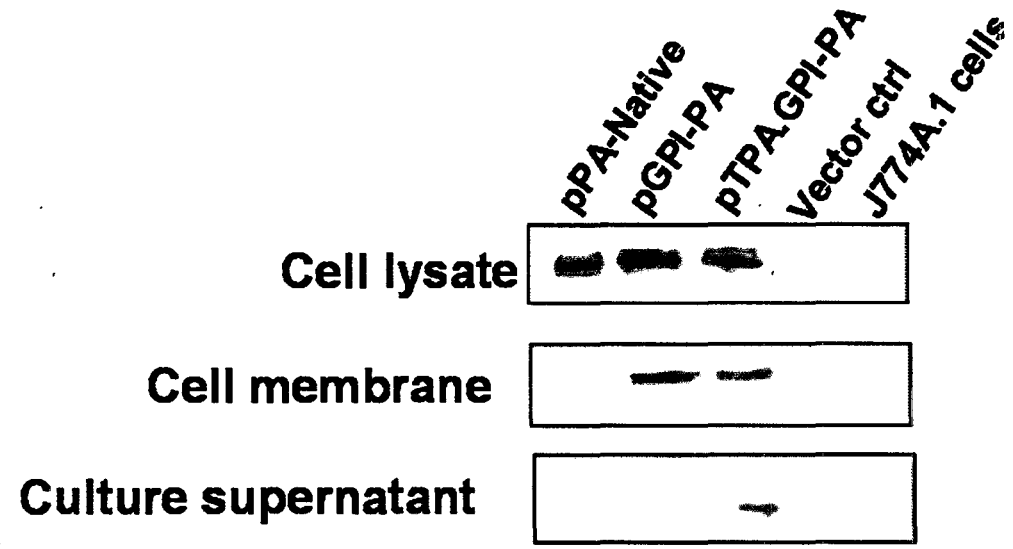


FIGURE.8B.

Figure.8 A & B. The address tags efficiently target PA63 to various sub cellular locations. Shows expression of different forms of protease-cleaved fragment of protective antigen in J774.A1 mouse MΦ-like cells. Cells were transfected with DNA constructs. Cell lysates and lysosomal fractions were prepared 36 hrs post-transfection. Alongwith that cell culture supernatant proteins were harvested by acetone precipitation. Subsequently, the protein samples were subjected to 12% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane. Blot was probed with anti-PA polyclonal serum followed by alkaline phosphatase-conjugated anti-rabbit IgG and developed using BCIP/NBT as substrate.

DISCUSSION

Delivery of naked DNA encoding antigens results in induction of adaptive immune responses (Berzofsky *et al.* 2001). This vaccination method has great potential in its ability to induce focused immune responses to defined antigens of different infectious diseases and tumors. It also has benefits in its ease of preparation and stability (Srivastava *et al.* 2003). Furthermore, DNA vaccination avoids the need for in vitro growth of virulent microorganisms, purification and modifications of protein/peptide preparations, a continuous "cold chain" (Srivastava *et al.* 2003), and circumvents the impact of pre-existing immunity to the carrier organism on vaccine efficacy. However, DNA vaccination has met with limited success likely due, at least in part, to the lack of strong elicited immune responses and in specific situations undesired polarized immune responses (primarily only Ab or CTL responses) (Srivastava *et al.* 2003; Dean *et al.* 2003).

For DNA vaccinations to have broad applications in both humans and animals, new approaches to delivery, adjuvant formulation, and the discovery of new Ags are needed. Eliciting stronger immune responses as well as inducing both humoral and cellular immunity is critically dependent on the antigen processing and presentation. Additionally, if successfully developed, would help generate protective immunity against infectious diseases, which are a serious public health problem in developing countries.

Despite improvements in treatment, anthrax remains a deadly infection. Primary prevention rests on creating a strong global norm that rejects development of such weapons. Secondary prevention implies early detection and prompt treatment of disease. Unfortunately, the tools of primary and secondary prevention are imperfect. Therefore, the currents scenario necessitates retrospection and potentiates the need for development of a vaccine that is beset with fewer side effects and can elicit protective immunity using a shorter dosage schedule in response to a bioterrorism event.

Keeping this information in mind, we addressed the question whether targeting the protease-cleaved fragment of protective antigen (PA63) to various subcellular locations with a set of signal sequences could influence the outcome of

an immune response in DNA vaccinated mice. For this we cloned the protease cleaved fragment of protective antigen (PA63) in DNA chimeras bearing a set of gene targeting sequences that were capable of targeting PA63 to various sub-cellular locations.

We first addressed the authenticity of the DNA chimeras and the ability of the address tags to target the encoded Ag to desired subcellular locations by transfection analysis followed by subcellular fractionation and immunoblotting. Results indicated that the PA63 protein along with the targeting signals was being properly recognized by the mammalian cells. LAMP1 signal successfully mediated the lysosomal targeting of PA63. On the other hand, TPA signal mediated the secretion of PA63 into the cell-culture supernatants. Combining these two signals lead to the lysosomal localization as well as cell-secretion of PA63. UQ signal however, solely encoded a cell-associated form of PA63. Hence, all the address tags successfully mediated the targeting of PA63 to various sub-cellular locations aimed at. Regardless of the signal sequence attached, all the constructs showed comparable expression levels.

Further, recombinant PA63 was expressed in *E.coli* M15 cells. An attempt was made to purify recombinant PA under denaturing conditions using metal affinity chromatography. Using this approach, 3 mg of protective antigen was purified to >95% from 1 litre culture. Analysis of the functional and biological activity of the purified recombinant PA63 expressed in *E. coli* indicated that the protein was biologically active and mediated its function as the receptor binding moiety of anthrax toxin complex in a manner similar to that displayed by full-length PA83 protein.

Having achieved the above two objectives, we proceeded to achieve the next objective of this study i.e. characterization of the cellular and humoral immune responses generated by these DNA chimeras, alone or followed by a recombinant PA63 protein booster, in mouse model.

CHAPTER 4

IMPACT OF TPA, LAMP1 & UBIQUITIN SIGNALS ON IMMUNE TARGETING

INTRODUCTION

Anthrax bioterror attacks of 2001 reinstated interest in *Bacillus anthracis*, the etiological agent of anthrax. Since then concerted efforts have been made in the direction of effective vaccine development, antitoxin-treatment strategies, and development of advanced diagnostics, for prevention, cure and detection respectively (Mock & Fouet 2001; Wang & Roehrl 2005). However, no significant breakthrough has been achieved as yet. Pre-exposure vaccination and post-exposure antibiotic therapy serve to be the only defense (Mock & Fouet 2001). Besides that the currently available anthrax vaccines suffer from the drawback, in being relatively reactogenic and having a long dosage schedule which requires frequent administration of boosters (Turnbull 2000; Scobie *et al.* 2003). Therefore, there is an urgent need to develop a safe, affordable and efficacious vaccine against anthrax.

Serious attempts have been made in the past to unveil the mechanism of host immune paralysis adopted by *Bacillus anthracis*. Gathered evidences point towards toxin-mediated immuno-modulation of host immune response, necrosis or apoptosis of various host cell types and, capsule-mediated inhibition of phagocytosis being the main immune evasion strategies of *Bacillus anthracis*. Cellular intoxication begins when PA (protective antigen, 83kDa), the receptor binding moiety binds to cell surface exposed anthrax toxin receptor on mammalian cells (Scobie *et al.* 2003; Bradley *et al.* 2001). Further upon furin cleavage, the protease-cleaved fragment of protective antigen (PA63) forms a pre-pore with a β-barrel structure, resulting from association of individual monomer loops (Klimpel *et al.* 1992, Ezzel *et al.* 1992; Milne *et al.* 1994). This pre-pore then binds to LF (Lethal factor) and EF (Edema factor) and the resulting complex gets subsequently internalized by endocytosis. Inside the endosome, pre-pore to pore conversion is triggered by the low pH in this compartment which ultimately mediates the translocation of LF and EF into the cytosol (Friedlander 1986; Mogridge *et al.* 2002; Abrami *et al.* 2003). Once inside cytosol, LF, a zinc metalloprotease, proteolytically cleaves short N-terminal fragments from mitogen or extracellular signal-regulated protein kinase kinasel (MEK1), MEK2 and MEK3, the upstream activators of ERK1, ERK2 and p38, respectively. As a result, antigen receptor signaling gets disrupted which causes

macrophage function inhibition (Duesbery *et al.* 1998; Vitale *et al.* 2000; Park *et al.* 2002) and suppression of T-lymphocyte activation (Paccani *et al.* 2005). On the other hand, EF, an adenylate cyclase, upon calmodulin binding undergoes structural rearrangements that leads to its activation and induces substantial increase in conversion of intracellular ATP to cAMP (Paccani *et al.* 2005). Subsequently, water homeostasis and cellular signaling of the host are disrupted, leading to edema during cutaneous anthrax infection (Leppla 1982 & 1984). Additionally, EF inhibits the ability of neutrophils to phagocytose the bacilli and produce oxidative burst (O'Brien *et al.* 1985; Wright & Mandell 1986). It also co-operates with LF to impair cytokine secretion during the infection of dendritic cells which ultimately leads to suppression of innate immunity (Cleret *et al.* 2006).

Therefore, the current scenario necessitates retrospection and design and development of an effective alternative approach for prophylaxis. In this context DNA vaccines seem to hold an enormous potential. Immunization with DNA vaccine encoding the antigen (Ag) of interest has been used to induce both cellular and humoral immunity. Reports of the first human clinical trials have shown that DNA vaccines are well tolerated and the results have been encouraging (Wang *et al.* 1998; Calarota *et al.* 1998; McGregor *et al.* 1998; Mulligan *et al.* 2006; Cebere *et al.* 2006). Although the immunological correlates of protection against anthrax have yet to be defined, several studies in non-human primates support the correlation between vaccine-induced neutralizing antibodies against PA and protection against subsequent challenge with pathogenic *B.anthracis* strain (Little *et al.* 1997; Turnbull *et al.* 1998; McBride *et al.* 1998; Pitt *et al.* 2001; Fellows *et al.* 2001). In addition, protection mediated by PA has been shown to be T-cell dependent (Williamson 1990 & 1999).

In this regard, DNA vaccination strategies have either relied on the usage of various adjuvants like, cation-lipids (Hermanson *et al.* 2004; Jimenez *et al.* 2007), CpG oligonucleotides (Klinman 2002); or else on DNA delivery by, gene-gun (Torres *et al.* 1999), liposomes (Sloat *et al.* 2006), poly-coglycoside particles (Ribeiro *et al.* 2007), with an aim to enhance the cellular as well as humoral immune responses. Systemic vaccination strategies based on prime boost regimen that imply a DNA prime followed by a booster with recombinant PA have been tested (Galloway *et al.* 2004; Hahn *et al.* 2004). Additionally, DNA vaccine encoding a

fragment of LF has been shown to provide protection against lethal toxin challenge (Price *et al.* 2001). Although these vaccine regimes were tested for their ability to induce measurable antibody responses, the generation of effective cellular adaptive immunity arising as a result of DNA vaccination was not investigated.

Given these considerations we report here the application of antigen trafficking to various compartments of the cell with an ultimate goal of improving the humoral as well as cellular immune responses. There is an increasing body of evidence to suggest that both $CD8^+$ and $CD4^+$ T cells are critical for the generation of an effective immune response against an intracellular pathogen. Although both recognize non-native forms of the antigen in association with major histocompatibility complex (MHC) molecules, the presentation of the antigen to these two lymphocytic populations occurs through distinct pathways (Catherine *et al.* 2002). For processing through the MHC II pathway the antigen needs to be targeted to the endosomal or lysosomal compartment (Nuchtern *et al.* 1990; Harding *et al.* 1993). The traditional pathway for antigen targeting to lysosomal compartment involves phagocytosis or endocytosis of the exogenous antigen or specific targeting to lysosomal pathway by attachment of lysosome targeting signals. Based on these observations we reasoned that a molecular approach that enroutes the antigen to the MHC II pathway might facilitate endogenous presentation to $CD4^+$ T cells. At steady state, immunoreactive LAMP1 (lysosome associated membrane protein) is highly enriched in the late endosomes and lysosomes (Honing *et al.* 1995; Wu *et al.* 1995). LAMP and MHC II are closely linked in their co-localization in MIIC (for MHC II containing compartments) (Drake *et al.* 1999). Also, TPA leader sequence (Tissue plasminogen activator) has been shown to facilitate the secretion of the encoded antigen (Liang *et al.* 2005). Therefore, its inclusion in DNA construct may help facilitate secretion of vaccine-encoded antigen that could well be taken up as an exogenous antigen by phagocytosis or endocytosis by APC and directly targeted to endosomal pathway. In addition to efficient antigen targeting and presentation of antigen via the MHC II pathway, effective $CD8^+$ T cell responses are dependent upon efficient processing through MHC I pathway alongwith the help provided by $CD4^+$ T cells (Castiglioni *et al.* 2005). For that we took advantage of the fact that carboxy terminus of UQ (Ubiquitin) is a substrate for a site-specific protease (Holowaty *et al.* 2003). Rationale was, ubiquitination should target the protein for

rapid cytoplasmic degradation by the proteasome and increase the availability of the antigenic peptides for presentation through the MHC I pathway (Rock *et al.* 1994). Subsequently, immune recognition should be reflected in the magnitude of Ag-specific $CD8^+$ T cell-mediated immune responses generated.

We therefore utilized a set of gene targeting sequences that are *(Table1)* capable of targeting PA63 to various sub-cellular locations. The results demonstrated that the magnitude of immune response was dependent on the cellular location of the expressed antigen and the processing through appropriate pathway. High end-point titers of anti-PA IgG antibodies were maintained until 22 weeks. These were paralleled by high avidity toxin neutralizing antibodies (TNA). Additionally, effective cellular adaptive immunity was generated in the systemic compartment. A significant correlation was found between survival percentage and post-challenge anti-PA titers (r=0.854) and TNA titers (r=0.895). Overall, immune kinetics pointed that differential processing through various compartments gave rise to qualitative differences in the immune response generated by various chimeras.

MATERIALS AND METHODS

DNA VACCINE PLASMIDS AND RECOMBINANT PROTEINS

The DNA chimeras bearing the address tags (*Table 1*), pPA63-Native: (GenBank accession no. EU249810), pTPA-PA63 (GenBank accession no. EU249808), pUQ-PA63 (GenBank accession no. EU249809), pPA63-LAMP1 (GenBank accession no. EU249806), pTPA-PA63-LAMP1 (GenBank accession no. EU249807) were then utilized for immunization experiments. Also, purified recombinant PA63 (rPA63) was utilized for protein boosters.

Table 1. DNA constructs and the targeting signals attached.

PLASMID NAME	EXPRESSED PRODUCT
pTPA-PA63	N-terminal TPA signal, and 63 kDa mature protein.
pPA63-Native	63 kDa mature protein.
pLAMP1-PA63	C-terminal LAMP1 membrane anchor and 63 kDa mature protein.
pUQ-PA63	N-terminal Ubiquitin leader and 63 kDa mature protein.
pTPA-PA63-LAMP1	N-terminal TPA signal, C-terminal LAMP1membrane anchor and 63kDa mature protein.

PLASMID DNA PREPARATION

Plasmid DNA was prepared from overnight cultures of transformed DH5α bacteria in Luria Bertani Broth (Amersham) plus 50 μg/ml kanamycin sulfate (Amersham) and processed by using Endo-free Giga kits (Qiagen, Valencia, CA).

VACCINATION AND LETHAL TOXIN CHALLENGE

Six- to eight-week-old female Balb/c mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA

suspended in phosphate-buffered saline (PBS; 50 µl per hind leg) administered via a 26-gauge, 1-ml hypodermic needle. All mice received the first DNA booster dose 28 days after their initial immunization and received either a second DNA booster (100µg, i.m.) or a s.c. injection of rPA63 protein (12.5 µg formulation with Incomplete Freunds Adjuvant) 28 days thereafter. Sera were obtained from blood samples collected at 4 wk intervals. For lethal toxin challenge, 50 µg of PA83 and 22µg of LF were injected intravenously via the tail vein, and the mice were closely monitored for 15 days.

ELISA DETECTION OF ANTI-PA REACTIVITY IN PLASMA

Anti-PA reactivity of the sera from immunized mice was determined by direct ELISA. Briefly, microtiter plates were coated with rPA63 (10µg/ml). Following blocking in 5% BSA and washes in PBS-Tween 20 (0.2%), plates were incubated with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgA HRP conjugate (Santacruz Biotechnology) for 1 h at 37°C. After washes in PBS-Tween 20 (0.2%), plates were developed using TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

DETECTION OF TOXIN NEUTRALIZING ANTIBODY TITERS (TNA) IN PLASMA

The protective effects of anti-toxin Abs were determined using a previously described assay (Staats *et al.* 2007) that measures their capacity to protect the J774A.1 mouse MΦ-like cell line from Letx. Briefly, J774A.1 MΦ cells (5×10^4 cells/well) were seeded in 96-well, flat bottom plates. After the cells reach 50-70% confluency, serum dilutions were added together with Letx (187.5ng/ml each of PA and LF) and incubated for 4h. After an additional incubation of 30 min after the addition of MTT (0.5mg/ml) (Sigma-Aldrich), the cells were lysed with acidic isopropanol (0.04-0.1N HCl in 90% isopropanol). The A_{450} of 100% viable cells was

calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

IN VITRO CYTOKINE PRODUCTION

Splenocytes from the immunized mice were incubated alongwith with rPA63 (10 μg/ml), ConA (1.0 μg/ml; Sigma-Aldrich), or alone in complete medium (RPMI 1640 medium with glutamine supplemented with 10% fetal calf serum, antibiotics, and 5 x 10^{-5} M β-2-mercaptoethanol) in triplicates in 24-well flat-bottom plates at 37°C, 5% CO_2. Cell culture supernatants were harvested at for 24 hrs post stimulation for IL-2, IL-4 and IL-12 cytokine measurement and, at 72 hrs for IL-10 and IFN-γ measurement. Cytokine-specific sandwich ELISA was performed using OptEIA kit for the specific cytokine (BD Pharmingen) according to manufacturer's protocols.

IFN-γ AND IL-4 ELISPOT ASSAYS

IFN-γ and IL-4 responses specific for PA were determined by mouse IFN-γ and IL-4- specific ELISPOT kit (BD Biosciences) as described by manufacturer's protocol. Briefly, multiscreen filtration plates (96 wells; Millipore, France) were coated overnight at 4°C with 4 μg/ml of rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen, San Diego, CA) and rat anti-mouse IL-4 antibody (clone 11 B 11) Thereafter, the plates were washed and blocked with complete medium. Serial twofold dilutions of the spleen cell suspensions from immunized mice were added to the wells starting at a concentration of $5x10^5$ cell per well and re-stimulated in vitro for 48 hrs with rPA63 (10μg/ml), ConA (1.0μg/ml) or in medium alone. After extensive washes, the plates were revealed by incubation with 4.0μg/ml of biotinylated rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen) and biotinylated rat anti-mouse IL-4 antibody (clone BVD6-24G2) followed by incubation with streptavidin-alkaline phosphatase (PharMingen). Finally, spots were revealed using BCIP/NBT (Sigma Aldrich) as the substrate. The number of IFN-γ and IL-4-producing cells was determined by counting the number of spot-forming

(SFU) in each well with the ELISPOT reader (IMMUNOSPOT, CTL technologies), and the results were expressed as numbers of SFU per 10^6 cells.

EVALUATION OF ANTIBODY-SECRETING CELLS (ASC) BY THE ELISPOT ASSAY

The splenocytes from immunized mice were re-stimulated in vitro as mentioned above. The number of PA-specific ASCs (IgA and IgG) were determined using biotinylated goat anti-mouse IgG or IgA (Sigma-Aldrich) and Alkaline phosphatase-labeled streptavidin-avidin (BD PharMingen) at a dilution of 1/800 in PBS-T following methods described previously (Boyaka *et al.* 2003). Results were expressed as number of ASC per 10^6 cells.

LYMPHOCYTE PROLIFERATION ASSAY

Spleen cells (3×10^5 cells) from the immunized mice were seeded in 96-well tissue culture plates (BD falcon). Cells were incubated with purified rPA63 protein (10μg/ml) and incubated at 37°C in 5% CO_2 atmosphere for 72 h. Cells stimulated with ConA (1μg/ml) and medium alone served as positive and negative controls, respectively. Proliferation was measured by MTT (Sigma-Aldrich) dye based-assay (Staats *et al.* 2007). Proliferation index (PI) was calculated as the ratio of the average O.D. value of wells containing antigen-stimulated cells to the average O.D. value of wells containing only cells with medium.

PREPARATION OF EFFECTOR T CELLS

Peptide-pulsed (synthetic overlapping peptides derived from PA sequence) spleen cells (mitomycinC treated) from Balb/c mice were taken as stimulator cells. Splenocytes from the immunized mice were resuspended in 2.0 ml of complete RPMI containing β-2-mercaptoethanol (5×10^{-5} M) and passed through nylon wool columns to enrich for T lymphocytes as described previously (Coligan *et al.* 1994) to separate non-adherent T cells. The cell number and cell viability were determined by trypan blue exclusion method. The enriched T cells were resuspended in complete RPMI and seeded in 24 well plates at a concentration of $0.4x10^6$ cells per well. Stimulator cells were also added to the wells at a concentration of $0.4x10^6$ cells

per well. The mix of enriched T cells and stimulator cells was then incubated for a period of 6 days at 37°C and 5% CO2. After 6-days coculturing, cell were collected and live effector cells were obtained by removing dead cells by Histopaque (1083) centrifugation as described previously (Esquifino *et al.* 1996).

MAGNETIC CELL SORTING

$CD4^+$ and $CD8^+$ T cells were purified by immunomagnetic methods (Martinez *et al.* 1996). Briefly, live T cells isolated by Histopaque column purification were incubated with MACS magnetic MicroBeads to which monoclonal antibodies against CD4 molecule (clone GK1.5; isotype, rat IgG2b) or CD8 molecule (clone 53-6.7; isotype, rat IgG2a) had been coupled (Miltenyi Biotec, Calif.) at the concentration of 10 µl of MicroBeads per 10^7 total cells for 15 min in a refrigerator at 4°C. The cells were then washed with PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin. Following passage of the cells through a steel wool column in a magnetic field, the positively selected $CD4^+$ or $CD8^+$ T cells were eluted out. The purity of the selected $CD4^+$ or $CD8^+$ T-cell population was above 92% as determined by flow cytometry using the appropriate specific monoclonal antibodies as described above.

PREPARATION OF TARGET CELLS

Peptide-pulsed J774A.1 (H-2^d) mouse MΦ-like cells were taken as target cells. Only J774A.1 cells were also included to check non-specific lysis.

CYTOTOXICITY ASSAYS

The target cells were incubated with effector cells obtained from vaccinated mice at different Effector: Target ratios (5:1, 10:1, and 15:1) for 16 hr. Neutral red uptake assay, a non-radioactive assay procedure (He *et al.* 2001) was followed to assay CTL activity. The percentage of specific lysis was calculated as (O.D. of control − O.D. of experimental group)/O.D. of control × 100.

STATISTICAL ANALYSIS

The experimental data were analyzed by software programs Sigma Plot 8.1, GraphPad Prism 5 or Excel (Microsoft) and were expressed as mean ± SE. The statistical significance of differences was analyzed by a two-tailed Students t-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A *P*-value of <0.05 determined was considered statistically significant. Differences in the survival were evaluated using log-rank analysis of the Kaplan-Meier curves. Correlation coefficients were determined by linear regression analysis.

RESULTS

DNA chimeras induced a strong and long lasting systemic anti-PA antibody response. To address the issue whether the targeting signals could deliver the DNA encoded antigen to the immune system and induce an efficient humoral response was assessed. Mice were immunized with DNA chimeras and were bled at 4 week intervals for over a period of 22 weeks, and sera were analyzed for the presence of anti-PA IgG1, IgG2a, IgG2b, and IgA antibodies by ELISA. Following i.m. administration, 100% of the mice developed significant IgG1 antibodies after the first booster dose ($p<0.05$, *Figure.1*), however, pronounced titers developed only after the second booster dose ($p<0.01$, *Figure.1*). Significantly higher anti-PA IgG1 titers (≈50,000) were observed following immunization with the construct encoding pTPA-PA63-LAMP1 (Protein boost) as compared to the native construct immunized group ($p<0.001$, *Figure.1*) and; other groups immunized with constructs bearing the address tags ($p<0.05$). Notably, pTPA-PA63 construct also generated significant titers (≈40,000), following a protein boost, as compared to the native group ($p<0.001$) and other groups that received constructs bearing the targeting signals ($p<0.05$). Mice immunized with pUQ-PA63, pTPA-PA63-LAMP1+pUQ-PA63, pLAMP1-PA63, pTPA-PA63+pUQ-PA63 and native constructs; generated moderate to high IgG1 anti-PA titers. Evaluation of the anti-PA IgA end point titers displayed no significant differences between various groups however; titers $\geq 10^3$ were maintained for over a period of 22 weeks (*Figure. 2*). Furthermore, evaluation of the titers of complement fixing antibody i.e. IgG2a, depicted that titers of $\geq 10^4$ developed in the group immunized with pTPA-PA63, pTPA-PA63-LAMP1 and pUQ-PA63 after the second boost (*Figure.3*). For rest of the groups the titers ranged between 10^3 and 10^4. A similar trend was also seen for anti-PA IgG2b antibody which also displayed titers ranging between 10^3 and 10^4 (*Figure.4*). No anti-PA reactivity was seen in the serum of mice immunized with PBS and corresponding vector (not shown in figure).

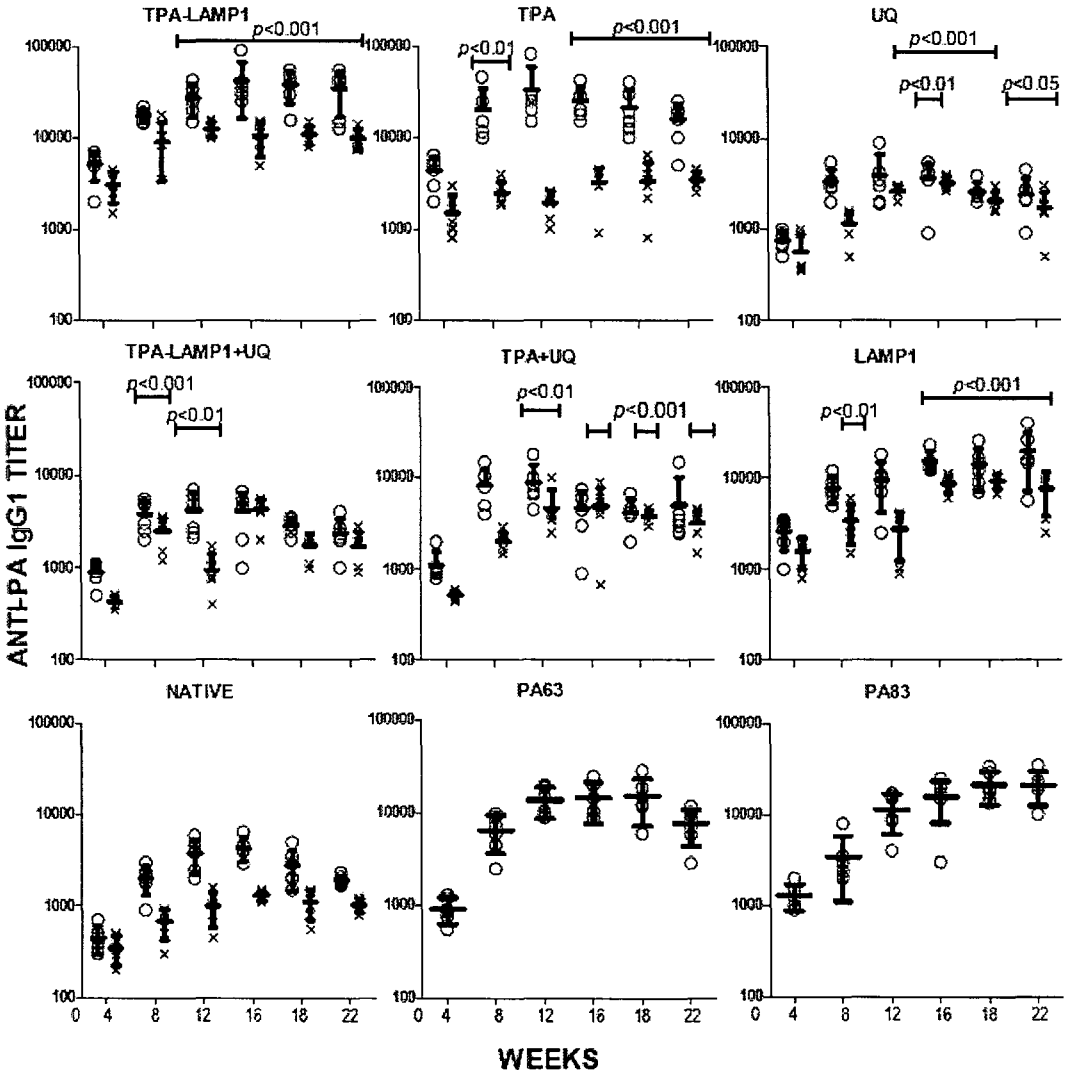


Figure.1. High anti-PA IgG1 titers were generated by DNA bearing TPA and LAMP1 tags. Groups of 6 Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG1 titers elicited following immunization were monitored till 22 weeks. Constructs pTPA-PA63-LAMP1 and pTPA-PA63 generated significantly higher anti-PA IgG1 titers after a protein boost as compared to the group pPA63-Native ($p<0.001$) and; other groups that received chimeras bearing the address tags ($p<0.05$). Individual anti-PA IgG1 titers, mean and 95% confidence level are shown for each group. Open circles represent titers obtained for mice that received a protein booster (**o**) and crosses (**x**) represent titers obtained for mice that received a DNA booster dose. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG1 titers.

We also examined the IgG subclass distribution as an indicator of the T-helper-cell subsets (TH1/TH2) induced by the heterologous prime boost strategy. For all the groups that received chimeras bearing TPA, LAMP1 address tags, the IgG1 titers

were approximately 1.0 log higher than the IgG2a responses which clearly indicated a TH2 bias. On the contrary, the chimera pUQ-PA63 displayed a TH1 bias on account of a lower IgG1: IgG2a ratio.

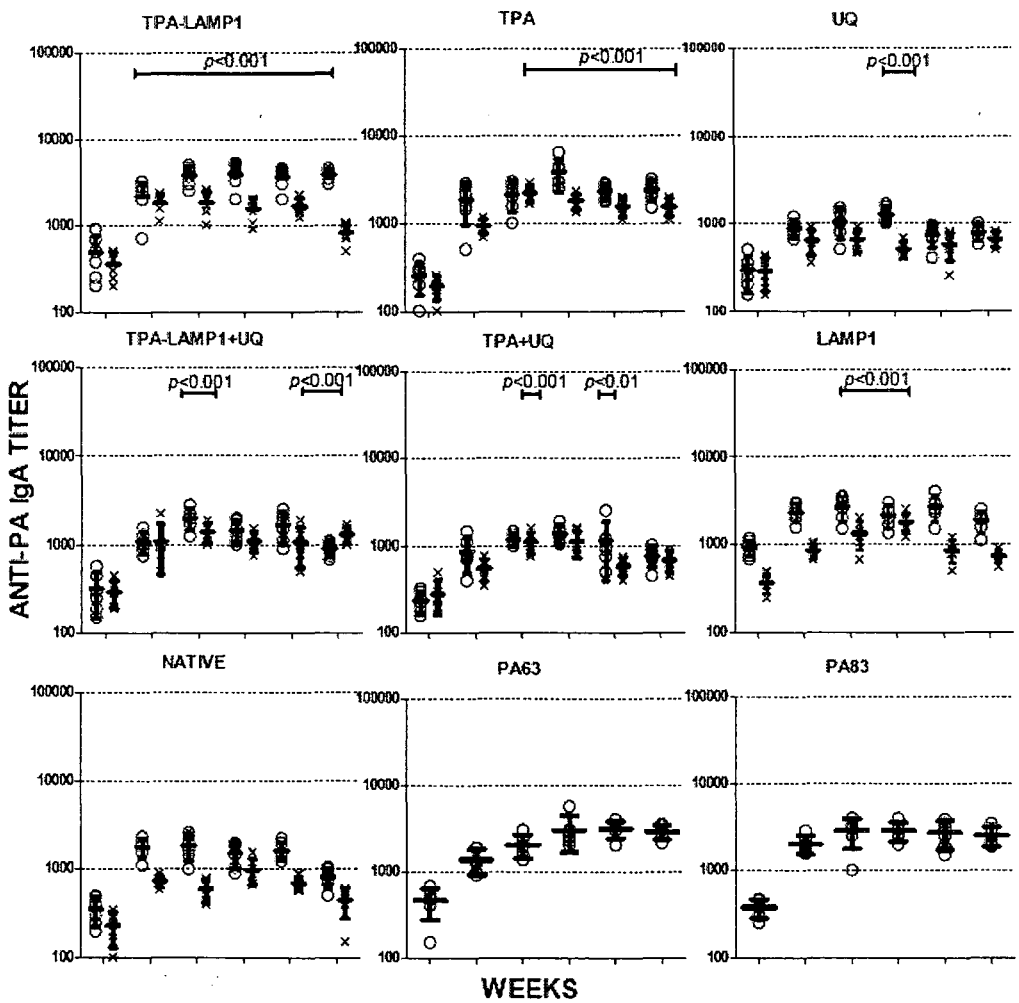


Figure.2. High anti-PA IgA titers were generated by DNA bearing UQ tag. Groups of 6 Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgA titers elicited following immunization were monitored till 22 weeks. Most of the groups generated significant IgA titers as compared to the group that received pPA63-Native construct ($p<0.05$). Individual anti-PA IgA titers, mean and 95% confidence level are shown for each group. Open circles represent titers obtained for mice that received a protein booster (**o**) and crosses (**x**) represent titers obtained for mice that received a DNA booster dose. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgA titers.

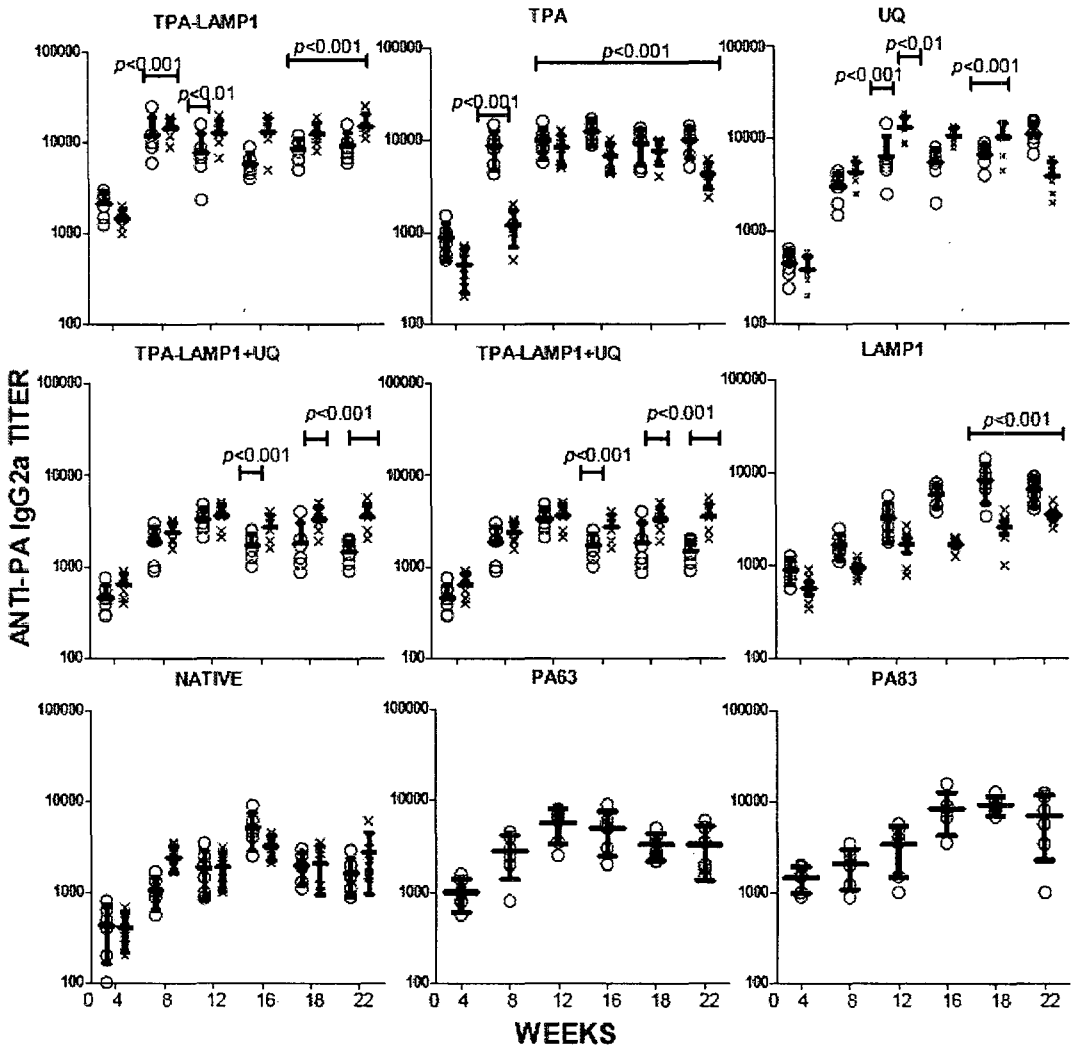


Figure.3. High anti-PA IgG2a titers were generated by DNA bearing UQ tag. Groups of 6 Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG2a titers elicited following immunization were monitored till 22 weeks. Most of the groups generated significant IgG2a titers as compared to the group that received pPA63-Native construct ($p<0.05$). Individual anti-PA IgG2a titers, mean and 95% confidence level are shown for each group. Open circles represent titers obtained for mice that received a protein booster (**o**) and crosses (**x**) represent titers obtained for mice that received a DNA booster dose. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG2a titers.

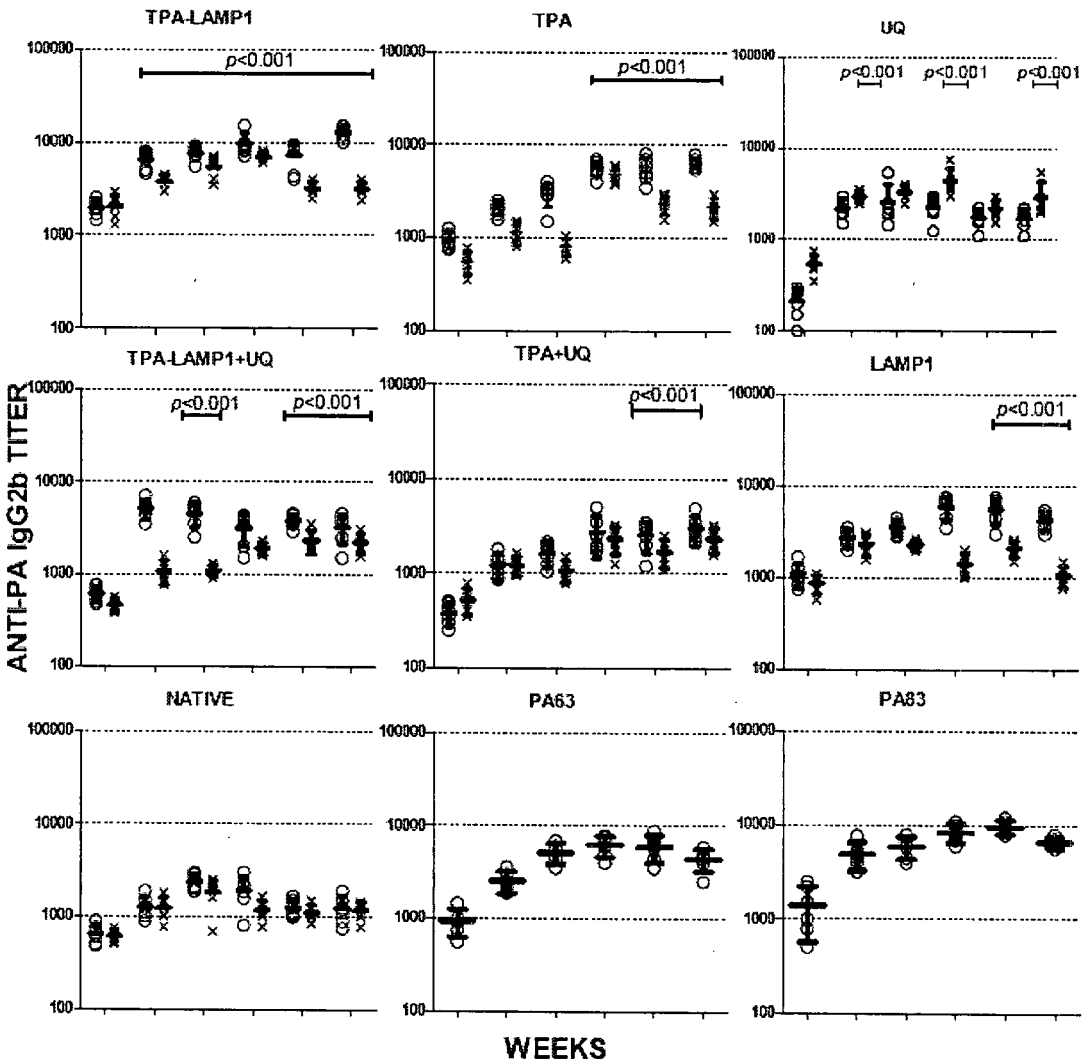


Figure.4. High anti-PA IgG2b titers were generated by DNA bearing UQ tag. Groups of 6 Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG2b titers elicited following immunization were monitored till 22 weeks. Most of the groups generated significant IgG2b titers as compared to the group that received pPA63-Native construct ($p<0.05$). Individual anti-PA IgG2b titers, mean and 95% confidence level are shown for each group. Open circles represent titers obtained for mice that received a protein booster (**o**) and crosses (**x**) represent titers obtained for mice that received a DNA booster dose. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG2b titers.

Efficient T-helper cell responses were generated after DNA vaccination. As cytokines play an important role in polarization of T-helper cell responses, we quantified the levels of type I (IL-2, IL-12, IFN-γ) and type II (IL-10, IL-4) cytokines secreted by splenocytes from immunized mice after re-stimulation in vitro with rPA63 (*Table.2*). IFN-γ: IL-10 ratio was taken as an indirect measure to determine the TH1/TH2 bias. The group immunized with pUQ-PA63 displayed a significantly higher IFN-γ: IL-10 ratio both after a DNA (17.42) and a protein boost (16.27) as compared to all other groups ($p<0.001$, Table.2). On the contrary the IFN-γ: IL-10 ratio for the group pTPA-PA63-LAMP1 was quite lower, 4.5 (Protein boost) and 5.11 (DNA boost). Another group, pTPA-PA63 also displayed significantly lower IFN-γ: IL-10 ratio (1.15 for DNA boost and 2.29 following protein boost1) as compared to the group pUQ-PA63. It was noteworthy, when mice were immunized with a combination of constructs pTPA-PA63 +pUQ-PA63, we observed a significantly higher IFN-γ: IL-10 ratio than when they were immunized with pTPA-PA63 alone (10.3 vs. 1.15 & 2.29, $p<0.01$). The construct pTPA-PA63-LAMP1+pUQ-PA63 also displayed a higher IFN-γ: IL-10 ratio as compared to pTPA-PA63-LAMP1 alone, however, the difference was not significant. The results therefore, depicted that TPA and LAMP1 signals biased the response towards TH2 type however; the bias was much more pronounced when these two signals were combined together as N- and C-terminal address tags. Contrarily, immunization with UQ bearing construct, alone or in combination, resulted in a preferential bias towards a TH1 type immune response.

Table 2. Cytokine production by spleen cells from the mice immunized with various DNA chimeras.

	CYTOKINE PROFILE [a]					
VACCINE COMBINATION	IL2[b),e)] (pg/ml)	IL12[b),e)] (pg/ml)	IFNγ[c),e)] (pg/ml)	IL4[b),e)] (pg/ml)	IL10[b),e)] (pg/ml)	TH1:TH2[d)]
pTPA-PA63 Pro	52.7 ± 5.5	1162.8 ± 25.5	1079.7 ± 104.1	6.62 ± 1.2	470.5 ±120.4	2.29
pTPA-PA63 DNA	43.3± 6.8	227.1 ± 59.9	89.9 ± 122.	13.41 ± 0.91	165.5 ± 12.3	1.15
pUQ-PA63 Pro	25.8 ± 5.7	135.2 ± 23.5	1600.2 ± 207.6	3.08 ± 0.87	98.35 ± 45.7	16.27[f)]
pUQ-PA63 DNA	9.72 ± 3.2	150.4 ± 21.6	1296.2 ± 89.7	3.41 ± 0.78	74.44 ±12.9	17.42[f)]
pTPA-PA63-LAMP1 Pro	90.9 ± 15.1	2770.9 ± 169.8	1251.8 ± 334.9	41.6 ± 5.9	277.7 ± 15.5	4.50
pTPA-PA63-LAMP1 DNA	27.8 ± 2.6	1970.9 ± 150.8	1003.12 ± 45.9	40.9 ± 9.0	196.3 ± 35.9	5.11
pPA63-LAMP1 Pro	61.5 ± 11.4	680.0 ± 109.8	177.08 ± 34.9	53.0 ± 12.5	108.3 ± 27.9	1.64
pPA63-LAMP1 DNA	19.8 ± 4.8	780.9 ± 100.8	186.04 ± 56.8	41.0 ± 12.8	135.5 ± 45.1	1.37
pPA63-NATIVE Pro	91.4 ± 19.1	133.8 ± 18.8	294.79 ± 40.9	3.45 ± 0.89	154.1 ± 22.7	1.91
pPA63-NATIVE DNA	18.4 ± 3.9	109.04 ± 25.8	263.54 ± 39.8	3.17 ± 0.99	96.38 ± 17.7	2.73
pTPA-PA63+ pUQ-PA63 Pro	74.5 ± 14.2	102.34 ± 33.8	887.7 ± 58.99	5.4 ± 1.5	85.8 ± 22.9	10.35
pTPA-PA63+ pUQ-PA63 DNA	25.5 ± 4.9	172.85 ± 22.9	900.12 ± 65.23	3.61 ± 1.1	87.22 ± 16.9	10.32[g)]
pTPA-PA63 -LAMP1+ pUQ-PA63 Pro	90.8 ± 11.8	840.95 ± 276.9	1111.2 ± 34.8	41.6 ± 5.6	143.3 ± 20.8	5.66[g)]
pTPA-PA63-LAMP1+ pUQ-PA63 DNA	4.54 ± 0.98	110.0 ± 25.1	208.98 ± 22.5	39.2 ± 9.9	134.1 ± 18.5	7.56
rPA63	55.7 ± 10.7	135.17 ± 35.9	94.79 ± 31.9	17.8 ± 3.8	88.88 ± 22.9	1.07
rPA83	128 ± 21.1	360 ± 57.8	122.2 ± 34.8	17.3 ± 4.7	81.66 ± 33.9	1.50
PBS	1.26 ± 0.23	1.95 ± 0.11	1.79 ± 0.34	1.01 ± 0.23	2.66 ± 0.33	0.67
VECTOR	1.28 ± 0.56	1.12 ± 0.25	1.10 ± 0.56	1.23 ± 0.34	1.23 ± 0.45	0.89

[a)] Groups of 8–10 mice were immunized i.m. with different DNA constructs. Four weeks post the last immunization mice were euthanized to take out the spleens and spleen cells from the mice were cultured in the presence 10μg/ml of rPA63 protein to determine the cytokine concentration in the cell-free supernatant.

[b)] Cell-free supernatants were collected at 24 h, for IL-2, IL-4 and IL-12 measurement.

[c)] Cell-free supernatants were collected 72 h later (for IFN-γ and IL-10)

[d)] Indirectly measured as IFN-γ: IL-10 ratio.

[e)] Each value represents mean ±SE of 4-6 mice tested in a group in three independent experiments.

[f)] Significantly higher ($p<0.001$) ratio compared to all the groups bearing TPA, LAMP1 address tags as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[g)] Significantly higher ($p<0.01$) ratio compared to the pTPA-PA63 group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Efficient B- and T-cell recall responses were generated in vitro. To measure the cellular immune responses elicited by DNA vaccination, splenocytes from immunized mice were isolated to determine PA-specific IL-4 and IFN-γ responses upon re-stimulation with rPA63 in vitro by ELISPOT (*Figure.5A & B*). Data shows that compared to the control group (immunized with vector and PBS) all other groups developed discernible PA-specific IFN-γ and IL-4 responses. We also included unstimulated and ConA (1.0μg/ml) stimulated splenocytes for all vaccination groups as negative and positive controls, respectively (not shown in figure).

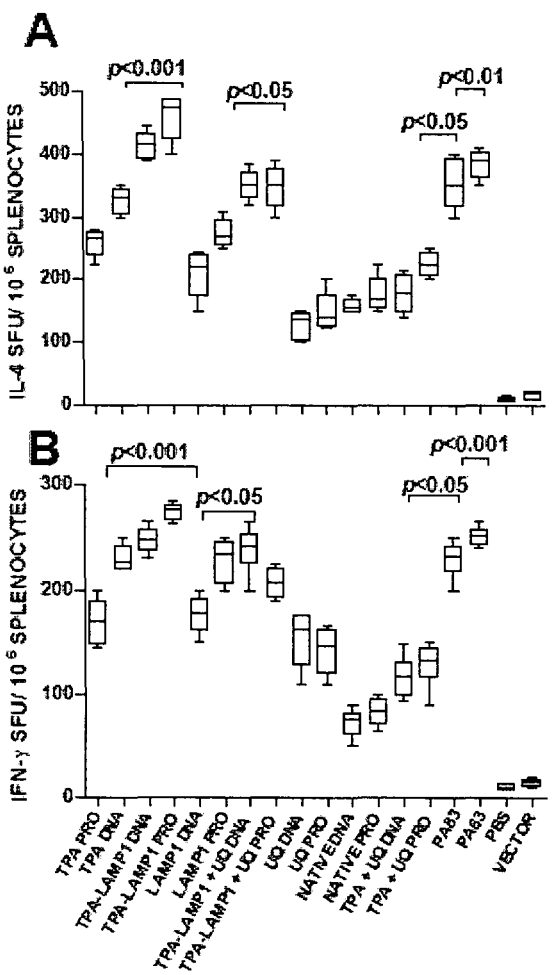


Figure. 5. A & B. Robust T-cell responses were generated upon restimulation with PA in vitro. Splenocytes from the immunized mice (n=6) were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 48 h. Figure shows the number of IL-4 **(A)** and IFN-γ **(B)** secreting cells as determined by ELISPOT assay. Results are expressed as mean SFU per group and 95% confidence level with $p<0.05$ vs. the group that received the pPA63-Native construct The mean number of spots in the presence of medium alone or ConA was ≤ 5.0 and $\geq 10^3$, respectively, and were not significantly different between the groups.

The level of SFU/10^6 splenocytes for the ConA stimulated splenocytes was $\approx 10^3$ whereas the number of SFU/10^6 splenocytes for unstimulated controls ranged between 0 and 5 and were not significantly different between the groups. The magnitude of IFN-γ secreting spleen cells for the groups that received pPA63-LAMP1, pTPA-PA63 (DNA boost) and pUQ-PA63 was found to be ≈200 SFU/10^6 splenocytes which was quite significant as compared to the group pPA63-Native

(p<0.01, *Figure.5B*). Also, the groups that received pTPA-PA63 (Protein boost), pTPA-PA63-LAMP1 (DNA boost) and its combination with pUQ-PA63, or else s.c. injections of rPA63 and rPA83 proteins, generated a significant number of IFN-γ SFUs; ≈250 SFU/10^6 (p<0.001, *Figure.5B*). However, the group pTPA-PA63-LAMP1 (Protein boost) displayed the highest IFN-γ SFU/10^6 of ≥250 (p<0.001 vs. pPA63-Native, *Figure.5B*).

We also quantified the PA-specific IL-4 secreting cells after vaccination. As can be seen in the *Figure. 5A*, the number of IL-4 secreting cells was significantly higher (≥400 SFU/10^6) in the group that received pTPA-PA63-LAMP1 than the group that received pPA63-Native construct (p<0.001, *Figure.5A*). Following closely were the groups that received s.c. rPA63 and rPA83 proteins (≤400 SFU/10^6). Rest of the groups that received TPA, LAMP1 bearing constructs along with their combinations with pUQ-PA63 developed ≤350 SFU/10^6 splenocytes. Overall, the group pTPA-PA63-LAMP1 (both DNA and protein boost) again displayed the highest SFU/10^6 level of ≥400 whereas the group pUQ-PA63 elicited the lowest count of IL-4 secreting cells (≥100 SFU/10^6). Of note, TPA, LAMP1 bearing chimeras generated an overall higher count of IL-4 secreting cells as compared to IFN-γ secreting cells (*Figure.5.A & B*). However, their combinations with pUQ-PA63 elicited an overall lower frequency of IL-4 SFUs as compared to when they were administered alone.

We also evaluated the number of IgG and IgA secreting cells in the total splenocyte population (*Figure.6A & B*). Enumeration of the IgG ASCs revealed that the results were in parallel with those obtained for IL-4 secreting cells. The group pTPA-PA63-LAMP1 elicited significantly higher number of IgG (≥600 ASC/10^6) antibody secreting cells as compared to the group that received native PA63 encoding chimera (p<0.001, *Figure. 6A*). Following closely were the groups that received recombinant protein immunizations which also elicited a significant count of ≈600 ASC/10^6 (p<0.001) as compared to the native group. Evaluation of the IgA ASCs detailed that the frequency of IgA ASCs for most of the groups was ≈300 IgA ASC/10^6 which was quite significant as compared to the pPA63-Native group (*Figure. 6B*). The results, therefore, pointed that LAMP1 and TPA address tags favor efficient recall B-cell responses upon re-stimulation with PA in vitro.

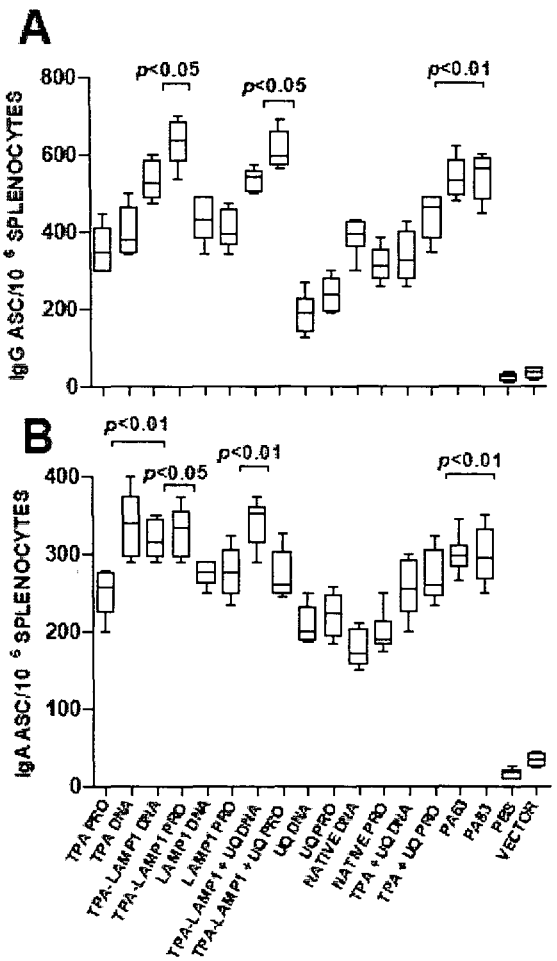


Figure. 6. A & B. Enhanced recall B-cell responses were induced by DNA vaccination. Splenocytes from the immunized mice (n=6) were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 48 h. Figure shows the number of IgG **(A)** and IgA **(B)** antibody secreting cells as determined by ELISPOT assay. Results are expressed as mean ASC's per group and 95% confidence level with $p<0.05$ vs. the group that received the pPA63-Native construct The mean number of spots in the presence of medium alone or ConA was ≤5.0 and $\geq 10^3$, respectively, and were not significantly different between the groups.

Induction of potent cellular proliferation responses on DNA chimera delivery. Spleen cells from the immunized mice were harvested 4 weeks after the last immunization and were re-stimulated for 3 days in the presence of for rPA63 (10μg/ml) for a standard MTT-based in vitro lymphocyte proliferation assay. As shown in *Figure.7*, pTPA-PA63-LAMP1 DNA construct displayed the highest proliferation index of ≥15.0 followed closely by the group that received pTPA-PA63 protein boost (PI ≈13). These PI values were quite significant as compared to the group pPA63-Native ($p<0.01$, *Figure.7*). Of note, the group pTPA-PA63-LAMP1+pUQ-PA63 also elicited a high PI ≈11.0 which was quite significant as compared to the native group ($p<0.05$, *Figure.7*). Other groups, namely pTPA-PA63 (DNA boost), pPA63-LAMP1, and the ones immunized with recombinant proteins also displayed a similar PI ≈11.0. Rest of the groups that received DNA constructs encoding PA with a UQ targeting signal alone or in combination also mounted a

significant PI ≤ 10. The results indicated that almost all the groups mounted strong proliferative responses indicating successful clonal expansion of PA-specific splenic cells on re-stimulation.

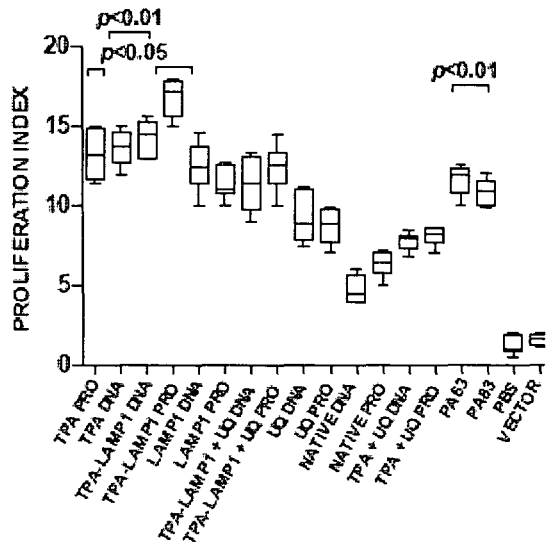


Figure. 7. A & B. Potent proliferative responses were generated by DNA vaccination. Splenocytes from the immunized mice were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 72 h. Proliferation was measured by a MTT (Sigma-Aldrich) dye based assay. The proliferation index (PI) was calculated from the ratio of the average O.D. value of wells containing antigen-stimulated cells to the average O.D. value of wells containing only cells with medium. Results are expressed as mean PI per group and 95% confidence level with $p<0.05$ vs. the group that received the pPA63-Native construct. No proliferation was seen in the un-stimulated controls.

Generation of potent cytotoxic T-lymphocyte responses following vaccination. In order to determine the contribution of specific T-cell populations to the observed CTL activity, after 6-day co-culturing of the stimulator cells and nylon wool enriched T cells derived from DNA immunized mice, the $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells were separated by a magnetic cell sorting method and tested for their individual abilities to lyse PA peptide-pulsed target cells. Results as depicted in *Figure.8.* indicate that $CD3^+$ $CD8^+$ T-cells elicited highest cell-cytotoxic potential in the group immunized with pUQ-PA63. These cells efficiently lysed peptide-pulsed target cells but did not lyse normal J774A.1 cells. On the contrary, $CD3^+$ $CD4^+$ T cells isolated from the groups

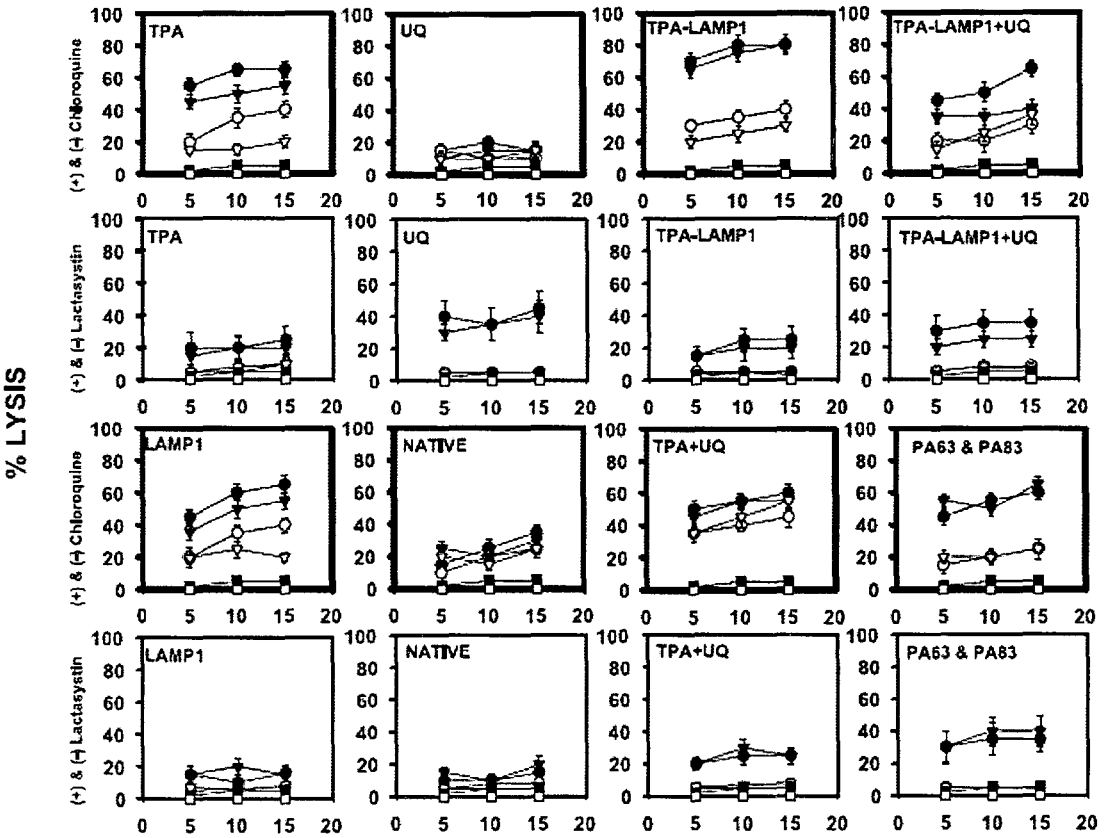


Figure.8. CD4$^+$ T-cell dependent cytotoxicity was generated by DNA chimeras bearing TPA, LAMP1 address tags whereas CD8$^+$ T-cell dependent cytotoxicity was displayed by DNA chimeras bearing UQ address tag. Effector CTLs were assayed for their ability to lyse target cells. Peptide-pulsed J774A.1 (H-2^d) MΦ-like cells were taken as target cells. Normal J774A.1 cells were also included to check non-specific lysis. Lytic ability of the effector CD4$^+$ and CD8$^+$ CTLs on antigen-pulsed target cells was evaluated at different Effector: Target ratios (5:1, 10:1, and 15:1). Neutral red uptake assay, a non-radioactive assay procedure [101] was followed to assay CTL activity. The percentage of specific lysis was calculated as (O.D. of control − O.D. of experimental group)/O.D. of control × 100. CTL activity of effector CTLs from mice (that received a protein boost) on PA-pulsed target cells is shown by filled circles and on normal J774A.1 cells as open circles. CTL activity of effector CTLs from mice (that received a DNA boost) PA-pulsed target cells is shown by filled triangles and on normal J774A.1 cells only as open triangles. Vector and PBS immunized control mice did not show any CTL activity (not shown in figure). Each curve represents mean CTL activity ±SE displayed by of 4-6 mice tested in a group in two different experiments.

pTPA-PA63-LAMP1 and pTPA-PA63 exhibited highest cell cytotoxic potential. Following closely were the groups immunized with rPA63 and rPA83 that also displayed predominantly CD3$^+$ CD4$^+$ T-cell mediated cytotoxicity. In contrast to the CD3$^+$ CD4$^+$ T cells, CD3$^+$ CD8$^+$ T cells isolated from these groups lysed significantly fewer peptide-pulsed target cells than did CD3$^+$ CD4$^+$ T cells. The

groups that received combinations of pUQ-PA63 with LAMP1 and TPA bearing chimeras elicited both, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cell cytotoxic responses however, the lytic ability of the former was higher than the latter. Overall, $CD4^+$ T-cell cytotoxicity was displayed by the groups immunized with chimeras bearing TPA, LAMP1 address tags whereas predominantly $CD8^+$ T-cell cytotoxicity was displayed by groups receiving UQ-bearing chimera.

Efficient toxin neutralizing antibody responses were developed following DNA vaccination.

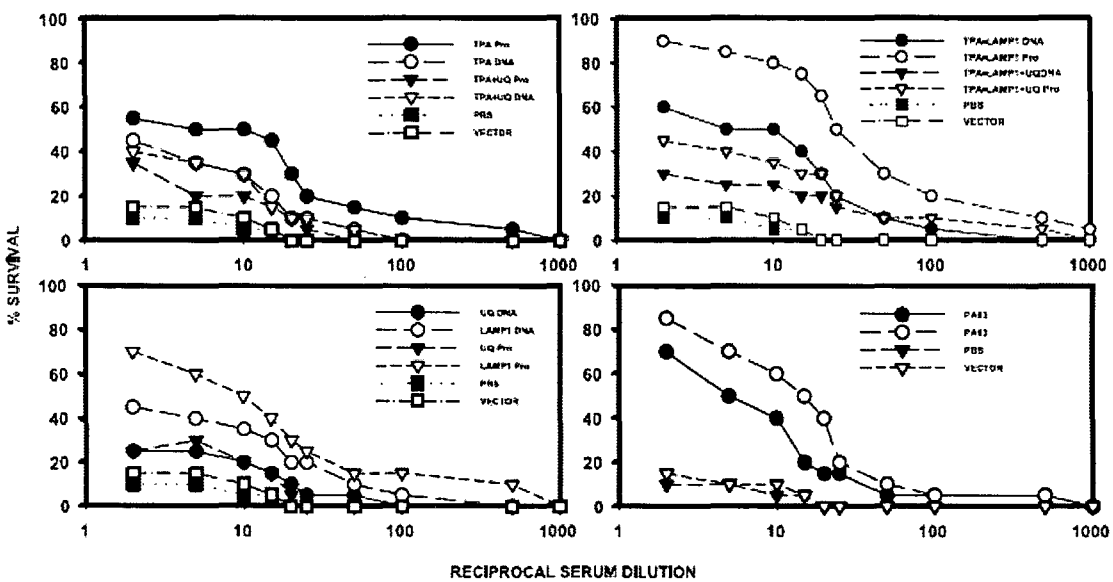


Figure.9. Efficient neutralization of lethal toxin by serum from immunized mice. Various dilutions of sera pooled from mice vaccinated with DNA vaccine constructs was pre-incubated with PA for 1 h. This mixture was added to J774.A1 MΦ-like cells in the presence of LF for 3 h, and cell survival measured. Results are expressed as percentage survival of J774.A1 MΦ-like cells following a lethal toxin shock and represent the average of two independent experiments.

Pre-challenge lethal toxin neutralizing antibody (TNA) titers were measured in the serum of all animals 2 weeks after each immunization. Neutralization activity was expressed as percentage survival of the J774.A1 MΦ-like cells following lethal toxin shock in the presence of anti-PA antibodies from the serum of immunized mice. For evaluation of TNA titers, we initially used PA and LF at a concentration of 1.0 μg/ml each. At this concentration the maximal pre-challenge TNA titers obtained for almost all the groups were ≤50 and we could not predict any significant

differences in the TNA titers elicited by various groups (*Figure. 9.*). The concentration of PA and LF utilized for this assay varies widely, with ranges 0.05 to 1.0μg/ml for PA and 0.01 to 1.0 μg/ml for LF (Little *et al.* 1990; Brossier *et al.* 2004; Hull *et al.* 2005; Lim *et al.* 2005). It has, however, been reported that a final concentration of 187.5ng/ml of PA and LF is 4-8 fold more toxin than what is needed to kill 100% of the J774A.1 MΦ-like cells (Staats *et al.* 2007). Therefore, for further evaluation we utilized a concentration 187.5 ng/ml each of PA and LF.

Reduction in the concentration of PA and LF revealed the variations in the toxin neutralization response developed by each group. Results also demonstrated that toxin neutralizing activity appeared in the serum of animals only after the first booster dose (*Table 3*). These antibodies could efficiently neutralize the lethal toxin action. The group pTPA-PA63-LAMP1 elicited the highest TNA titers of ≥600 for both protein and DNA boost. These titers were significantly higher than the pPA63-Native group ($p<0.001$, *Table 3*). Groups that received pTPA-PA63 and pPA63-LAMP1 also elicited significant TNA titers ranging from 350-450 ($p<0.001$ and $p<0.01$, respectively, vs. pPA63-Native).

Contrarily, the group pUQ-PA63 elicited the lowest TNA titers of ≈100 indicating low toxin neutralizing response. The chimeras, pTPA-PA63 and pTPA-PA63-LAMP1 elicited significantly higher TNA titers as compared to their combinations with pUQ-PA63 ($p<0.05$, *Table3*). Sera from control mice did not neutralize anthrax lethal toxin at all. Overall the results indicated that TNA titers were significantly higher in the serum of animals immunized with chimeras bearing both the TPA and LAMP1 address tags than the animals immunized with the chimeras bearing them independently or lacking them.

Table 3. Pre-challenge TNA titers in the serum of immunized mice.

TNA titers [a], 10^3 ±SE		
Construct	**I[st] Boost**	**2[nd] Boost**
pTPA-PA63 Pro	0.325±0.08	0.450±0.10 [b), d)]
pTPA-PA63 DNA	0.230±0.07	0.390±0.09 [d)]
pUQ-PA63 Pro	≤0.05	0.110±0.02
pUQ-PA63 DNA	≤0.05	0.101±0.01
pTPA-PA63 -LAMP1 Pro	0.475±0.10	0.650±0.10 [b), d)]
pTPA-PA63 -LAMP1 DNA	0.410±0.09	0.625±0.19 [b), d)]
pPA63-LAMP1 Pro	0.290±0.07	0.400±0.15 [c)]
pPA63-LAMP1 DNA	0.250±0.10	0.350±0.10
pPA63-NATIVE Pro	≤0.05	≤100
pPA63-NATIVE DNA	≤0.05	≤100
pTPA-PA63 +UQ-PA63 Pro	0.215±0.08	0.365±0.09
pTPA-PA63 +UQ-PA63 DNA	0.200±0.09	0.305±0.08
pTPA-PA63 -LAMP1+ UQ-PA63 Pro	0.110±0.02	0.245±0.05
pTPA-PA63 -LAMP1+ UQ-PA63 DNA	0.100±0.04	0.205±0.09
rPA63	0.250±0.05	0.395±0.10 [c)]
rPA83	0.310±0.10	0.425±0.10 [c)]

[a)] Mice were immunized at days 0, 28 and 56; and TNA titers were measured in the serum of the immunized mice 14 days after each immunization. Titers after priming were ≤50 for all the groups. Corresponding values after I[st] and 2[nd] booster dose represent TNA titers obtained for of 4-6 mice tested in a group in three independent experiments and results are expressed as mean ±SE.

[b)] Significantly higher (p<0.001) titers as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c)] Significantly higher (p<0.01) titers as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d)] Significantly higher (p<0.05) titers as compared to those groups that received their combinations with pUQ-PA63 chimera as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

DISCUSSION

Major drawbacks associated with the currently licensed anthrax vaccines potentiate the need for development of a vaccine that is beset with fewer side effects and can elicit protective immunity using a shorter dosage schedule in response to a bioterrorism event. In this context, there is a broad consensus that DNA vaccines can serve as an attractive target. The ability of DNA vaccines to provide effective immunological protection against infection rests on the generation of efficient $CD4^+$ and $CD8^+$ T cell responses which requires presentation of the vaccine-encoded antigen in context with MHC I and MHC II molecules, respectively. These molecules engage different antigen-processing pathways namely, cytosolic for MHC I and endosomal for MHC II molecules.

It is, therefore, likely that the outcome of an immune response can be influenced by the form of antigen administered and its ability to access various Ag-processing pathways. This would in turn be revealed by the spectrum of B- and T-cell responses represented by initial clonal outburst, polarization of T-helper cell responses (TH1/TH2), antibody isotype switch and B- and T-cell memory responses. Keeping this information in mind, we addressed the question whether targeting the protease-cleaved fragment of protective antigen (PA63) to various subcellular locations with a set of signal sequences could influence the outcome of an immune response in DNA vaccinated mice. Our results demonstrated that it does indeed influence the immune outcome as manifested by the induction of high end point anti-PA titers, high-avidity toxin neutralizing antibodies, and potent cellular proliferative and cytotoxic T lymphocyte responses.

Since the heightening of immune responses appeared to be rooted in the differences in the targeting signals attached, we first examined the nature of anti-PA IgG antibody subclass induced by DNA vaccination. The pattern of IgG subclass is known to mirror the T-helper cell-derived cytokine responses (Finkelman *et al.* 1990) and different IgG subclasses (i.e. the complement fixing IgG2a Abs vs. non-complement fixing IgG1 and IgG2b) are involved in distinct mechanism of host protection (Spiegelberg *et al.* 1989). All these facts argue for detection of anti-PA IgG subclass pattern to predict the correlates of protective immunity. Analysis of the

end-point titers of IgG1 antibody indicated that titers of highest magnitude were elicited by the group immunized with pTPA-PA63-LAMP1 (both DNA and protein boost). Not only that, this group also mounted high end-point titers of complement fixing IgG2a antibody. Close examination of IgG subclass distribution indicated a higher IgG1:IgG2a ratio whenever the groups were immunized with chimeras bearing TPA, LAMP1 signals alone or when they were combined together in a chimera as N- and C-terminal address tags. Importantly, electron microscopic studies have shown that spore-associated proteins can be recognized by anti-PA antibodies, and PA-immune serum from several species enhanced the phagocytosis of spores of the virulent Ames and Sterne strains by murine peritoneal macrophages (Welkos *et al.* 2001 & 2002). In addition, it has been shown that IgG1 subclass displays highest affinity towards PA (Little *et al.* 1988). Therefore, development of high end-point titers of IgG1 antibody upon DNA vaccination gains high merit from anti-spore activity point of view. It is also noteworthy that these high end-point titers were maintained until 22 weeks. Maintenance of such high end-point anti-PA titers can aid in controlling pathogen spread and potentially in reducing disease progression. Thus, high end-point titers of anti-PA antibodies arising as a result of targeted DNA vaccination can overall act to, (1) enhance sporicidal activities of macrophages (Welkos *et al.* 2001 & 2002); (2) impede spore germination in vivo (Little *et al.* 1988; Cote *et al.* 2005) and; (3) enhance the ability of the early responding cells to ablate infection (Cote *et al.* 2005).

There also exists compelling evidence that toxin neutralizing antibodies afford protective immunity (Reuveny *et al.* 2001; Weiss *et al.* 2006; Peachman *et al.* 2006). The functional significance of the development of high TNA titers gains considerable importance in light of the fact that death of the infected animal occurs due to accumulation of lethal level of toxin although the *Bacillus* itself is sensitive to anti-bacterials (Gold *et al.* 1967). Therefore, we assayed the pre-challenge TNA titers to predict any correlation between the presence of toxin neutralizing antibodies in the serum and protection against lethal challenge. However, analysis of TNA titers emphasized that the conditions utilized in the performance of toxin neutralization assay, like the concentration of PA and LF, are very important for the ability of the assay to correlate with protection in vivo. No significant titers could be detected when a high Letx dose (1.0 μg/ml each of PA and LF) was taken.

Evaluation of TNA titers in the presence of 187.5ng/ml of Letx (4-8 fold toxin dose) revealed the variations in the titers elicited by different vaccination groups. Various other studies have also pointed the importance of the assay conditions, like, prior incubation of toxin with anti-PA serum before subjecting the MΦ cells to a lethal shock (Turnbull *et al.* 1988; Little *et al.* 1997; Jimenez *et al.* 2007), and passive transfer of antibody before injecting Letx (Staats *et al.* 2007; Albrecht *et al.* 2007); to determine toxin neutralizing activity in vitro and in vivo, respectively. Most importantly, high toxin neutralizing activity appeared in the serum of the immunized animals after the first booster dose which was paralleled by the generation of high avidity antibodies. Further rise in avidity of the antibodies towards PA, post-challenge, depicted affinity maturation. Since high avidity antibodies are conducive for rapid neutralization of anthrax toxin, these antibodies can well act against the toxin secreted by *Bacillus anthracis*. Taken together, these Letx neutralization antibodies and anti-PA antibodies generated upon immunization with various DNA chimeras can provide a parallel line of defense against the toxemia as well as bacteremia.

Although the production of antibody is necessary for protection against anthrax infection, it is recognized that a cell-mediated component is also required (McBride *et al.* 1998; Friedlander *et al.* 1999). Furthermore and consistent with this observation is the well-documented fact that effective cellular adaptive immunity requires the co-stimulatory signals and cytokines released by antigen-activated T-helper subsets (Kaech *et al.* 2002). In this regard, we evaluated the cytokine secretion profile of the T-helper cell subsets arising as a result of DNA vaccination. Our results clearly indicated that differential targeting was responsible for T-helper cell differentiation. Dissection of the cytokine secretion pattern indicated the generation of primarily TH2 biased responses on account of the attachment of the signals LAMP1, TPA or both (as measured indirectly from the IFN-γ: IL-10 ratio). Contrarily, inclusion of UQ signal in the DNA chimera resulted in a preferential bias towards TH1-type of immune response. Characterization of PA-specific effector T-cell responses (enumeration of cytokine secreting cells and T-cell mediated cytotoxicity) indicated the generation of effective cellular adaptive immunity in the systemic compartment. These results are very significant in light of recent studies that have pointed the role of IFN-γ producing $CD4^+$ T lymphocytes in protection

against virulent challenge (Glomski *et al.* 2007). Overall, the idea to target PA to both endocytic and cytosolic compartments was successfully exploited to generate efficient effector T-cell responses.

However, our findings are quite striking in view of the fact that all the vaccine combinations (5 out of the 7 tested) bearing the signals LAMP1 and TPA alone or together, aided the generation of TH2 biased responses as indicated by indirect IFN-γ: IL-4 and IgG1:IgG2a ratios. Further characterization of PA-specific effector B-cells indicated potential plasma IgA and IgG secretion. DNA vaccines have long been known for their capability to elicit TH1 biased responses favoring potent cytotoxic T-lymphocyte responses and weak in their ability to generate efficient humoral immunity (Yasutomi *et al.* 1996; Shiau *et al.* 2006). However, our study demonstrates high levels of plasma IgG and IgA secretion along with the generation of effective cellular adaptive immunity. In addition these responses were found to be at par with recombinant PA63/83 vaccinations (formulated with aluminum hydroxide adjuvant). Of note, anthrax is primarily a systemic disease resulting from the release of anthrax toxin in peripheral lymph nodes and in the general circulation (Dixon *et al.* 1999; Mock & Fouet 2001). Therefore, development of effective cellular and humoral immunity in the systemic compartment is of immense importance.

How do these signals mediate such a large impact on the immune outcome- was the next question we addressed? Analysis of the capacity of the processing compartments to generate peptides threw some light on this aspect. Processing of antigens can occur potentially through two pathways i.e., proteasomal degradation of the endogenous antigen and TAP transport to ER followed by binding to MHC I (Rock *et al.* 1994) or else degradation and loading of the exogenous antigen within the endosomes leading to MHC II loading (Nuchtern *et al.* 1990; Harding *et al.* 1993). Furthermore, within the endosomal pathway different endocytic compartments are involved in generating different peptides (Harding 1996). The LAMP1 protein resides mainly in lysosomes with minor amounts present in late endosomes as well. These compartments are defined by the differences in the accessibility to different endocytic tracers and pH (Mellman 1990). Differential processing in these compartments can therefore, give rise to both qualitative and quantitative differences in the generation of peptides from these compartments

(Fernandez *et al.* 2000). That probably explains why we observed differences in the magnitude and type of immune responses generated by the chimeras bearing TPA and LAMP1 signals although both of them targeted the antigen to endosomal pathway.

Of note, it is not only the targeting of the antigen to a pathway that results in an effective immune response but the generation of specific epitopes that confer protective immunity (Bailie *et al.* 2004; Albrecht *et al.* 2007). This probably was attained by attaching the signal sequences, LAMP1 and TPA, as N- or C-terminal address tags. Naturally, PA is secreted during an infection and undergoes structural rearrangements inside the endosome triggered by the low pH in this compartment (Dixon *et al.* 1999; Mock & Fouet 2001; Abrami *et al.* 2003). Therefore, targeting of PA63 to endosomal/ lysosomal compartments by DNA vaccination on account of the attachment of TPA and LAMP1 signals, alone or in combination, probably came closer to the generation of antigenic determinants that were needed for the generation of a better T-helper, cytotoxic and neutralizing antibody response. Our observation that generation of optimal epitopes is an absolute necessity to confer protective immunity receives support from earlier studies that have shown that monoclonal antibodies corresponding to specific epitopes are protective against anthrax lethal challenge (Peterson *et al.* 2007). However, generation of a response of highest magnitude, when these two signals were combined together, can be attributed to the attainment of a synergism in giving rise to optimal, specific, protective epitope peptides from both the endosomal/lysosomal compartments. This synergism was probably not attained when these two signals were present singly.

Inability of the UQ bearing chimera to generate protective immunity alone or in combination with TPA and LAMP1 bearing constructs could be as a result of the lack of optimal epitope generation on account of enhanced proteasomal degradation. Reports suggest that sustained protein expression may lead to an increased antigenic load and enhanced immunogenicity (Brulet *et al.* 2007). Although rapid proteasomal degradation of PA should lead to enhanced presentation by MHC I molecules, it may prevent the protein to reach the extracellular space and thus the APC surrounding the infectious site. In addition, the heightening or depreciation of an immune response is very likely dependent upon the dynamic relationship between the encoded antigen and the immune system. For maximal effect the antigenic

epitopes derived must be presented to the immune system at a certain rate, concentration and order (Turnbull *et al.* 1986). It is possible that the lack of stability of the PA63 protein expressed by pUQ-PA63 prevented any kind of synergism, in giving rise to optimal epitopes with those arising from TPA and LAMP1 bearing chimeras to generate an additive effect. However, the role of protein stability vs. immune response generation would require further analysis to evaluate its overall impact. Overall, all these factors contributed to the generation of heightened responses for the groups bearing TPA, LAMP1 signals as compared to the one bearing UQ signal.

Apart from this, the combinations that received protein boosters also mediated heightened responses as compared to the groups that received a DNA booster. Prime-boost (priming with DNA and boosting with protein) has been accredited to be a well known strategy for boosting immune responses (Price *et al.* 2001; Hermanson *et al.* 2004; Galloway 2004; Hahn *et al.* 2004; Gu *et al.* 1999). However, the mechanism by which a protein booster amplifies CTL or antibody responses is relatively unclear. Increased antigen availability at the crucial stage of boosting might be an explanation. It is known that for antibody induction, in addition to an effective T-cell help, more available antigen is provided by boosting for uptake by B cells, a factor favoring B-cell activation (Farr & Dixon 1960; Zinkernagel *et al.* 1997). Time taken for antigen expression followed by its uptake by B cells can, therefore, be a crucial parameter in boosting immune responses. That might explain why boosting with protein was more effective at raising specific antibody levels than DNA alone. However, a more striking result was evident in our prime-boost setting, i.e. the chimera pTPA-PA63-LAMP1 afforded equivalent protection both after DNA or a protein boost (88%) which indicated that the protective effect was maximal when the two signal sequences, TPA and LAMP1, were combined together as N- and C-terminal tags. Thus, the results pointed that immune outcome showed a greater dependence on targeting of the antigen to appropriate cellular compartment (s) rather than on prime-boost regimen.

Another aspect that was intriguing was the surprising better performance of our DNA chimera pTPA-PA63-LAMP1 over s.c. recombinant protein vaccine preparations (rPA63/83 aluminum hydroxide formulation). While it is very much established that recombinant protein vaccine preparations fare better than DNA

vaccine preparations, their combinations with various adjuvants can stimulate diverse immune responses. Studies have shown that guinea pigs inoculated with rPA/Ribi adjuvant were completely protected against a lethal challenge but were poorly protected when vaccinated with either rPA/alhydrogel or the licensed UK human anthrax vaccine (McBride *et al.* 1998) which is an aluminum hydroxide adsorbed supernatant material preparation (mainly PA) from fermentor cultures of toxigenic, non-encapsulated isolate of *B.anthracis* V770-NP1-R. Also, it was found difficult to protect guinea pigs by vaccination with aluminum-containing human vaccines (Pitt *et al.* 2001). Thus, the vaccine-adjuvant combinations may or may not confer protection and these responses further vary between species (Vogel *et al.* 1995).

Thus far, clinical studies of anthrax vaccine evaluation have relied only on determination of seroconversion and the contribution of the cellular adaptive immunity has not been thoroughly investigated. However, a recent study has suggested the role of IFN-γ producing $CD4^+$ T lymphocytes in protection against anthrax (Glomski *et al.* 2007). Importantly, such a protective IFN-γ pathway might only be functional when Letx is absent or neutralized by anti-toxin antibodies as Letx has been shown to inhibit $CD4^+$ T lymphocyte activation by disrupting antigen-receptor signaling (Paccani *et al.* 2005; Fang *et al.* 2005; Comer *et al.* 2005). In such a scenario the generation and maintenance of high end-point anti-PA titers and TNA titers alongwith the generation of effective cellular adaptive immunity in the systemic compartment can provide a parallel line of defense against the pathogen as well as the toxin and embody the paradigm of antibacterial and antitoxic components in a DNA vaccine preparation. Thus, we envision that the pTPA-PA63-LAMP1 can represent a prototypic, safe and efficacious DNA chimera for further evaluation and development of DNA vaccine against anthrax.

CHAPTER 5

IMPACT OF GPI & TPA SIGNALS ON IMMUNE TARGETING

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, has developed an ingenious strategy to target cells by secreting a soluble proteinaceous exotoxin which belongs to a large family of binary (AB type) pore forming toxins. The brisance caused by this binary toxin sets in once the 'B' subunit (Protective antigen; PA83) binds cell surface anthrax toxin receptor (ATR), TEM8 and CMG2 (Bradley *et al.* 2001; Scobie *et al.* 2003). Further upon furin cleavage, B subunit (PA63) forms a pre-pore with a β-barrel structure, resulting from association of individual monomer loops (Klimpel *et al.* 1992; Milne *et al.* 1994). Pre-pore to pore conversion requires the endocytosis of this β-barrel structure which undergoes structural rearrangements triggered by the low pH in the endosome to form a membrane spanning pore (Miller *et al.* 1999; Collier 1999).

All this is undertaken to ensure the translocation of the catalytically active 'A' subunit (Edema factor; EF and Lethal factor; LF) into the cytosol where it can hijack the cellular machinery (Turk 2007). Interaction with EF and LF with PA63 occurs at the cell surface after the heptamerization has occurred (Mogridge *et al.* 2002). Once in the cytosol, EF, an adenylate cyclase, upon calmodulin binding undergoes structural rearrangements that leads to its activation and induces substantial increase in conversion of intracellular ATP to cAMP (Leppla 1982). Subsequently, water homeostasis and cellular signaling of host are disrupted, leading to edema during cutaneous anthrax infection (Leppla 1982 & 1984). LF, on the other hand, a zinc-dependent metalloprotease, cleaves short N-terminal fragments from mitogen or extracellular signal-regulated protein kinase kinasel (MEK1), MEK2 and MEK3, the upstream activators of ERK1, ERK2 and p38, respectively (Duesbery *et al.* 1998; Vitale *et al.* 2000; Park *et al.* 2002) that disrupts antigen receptor signaling resulting in macrophage function inhibition and cell death (Park *et al.* 2002; Paccani *et al.* 2005).

The depredation thus, resulting from anthrax toxin action in affected individuals has riveted attention. Early treatment is essential, as the disease shows a rampant aggression at which antibiotics are no longer effective owing to the accumulation of a lethal level of toxin, even though the organism is sensitive to the agent. Post

exposure prophylaxis is a combination of an antibiotic (ciprofloxacin) and a licensed human vaccine AVA (Anthrax Vaccine Absorbed). But AVA suffers from the chief criticism of incomplete characterization of the actual composition of the vaccine preparation which often results in reactogenicity and generates the need for frequent administration of boosters (Wasserman *et al.* 2003). Therefore, the current scenario necessitates retrospection and search for a better and; safer anthrax vaccine.

Vaccination strategies have aimed at abrogating initial events of anthrax toxin action such as binding of PA to ATR, with an aim to counteract the disease before its inception (Yan *et al.* 2008). Studies have shown that ATR resides in the glycerolipidic i.e. non-raft regions of the plasma membrane. However, upon binding and heptamerization of PA, the toxin-receptor complex associates with lipid-raft like domains and undergoes rapid endocytosis via clathrin-dependent pathway (Abrami *et al.* 2003). This process subsequently leads to the translocation of the enzymatic subunits into the cytosol through the protective antigen pore. This kind of behavior parallels B-cell receptor which also undergoes ligand-dependent clustering and raft association (Cheng at al. 1999), and is subsequently internalized via clathrin dependent mechanism (Stoddart *et al.* 2002).

The observation that ATR couples raft translocation and mediates endocytosis of EF/LF along with the oligomerization of PA made us hypothesize that a DNA chimera encoding protease-cleaved fragment of PA (PA63) attached to a C-terminal glycosylphosphatidyl inositol (GPI) anchor sequence might ensure lateral association of the cell-surface GPI-anchored PA with liquid-ordered, cholesterol- and sphingolipid-rich domains or rafts. Biochemical, morphological and functional approaches have been able to trace that GPI-anchored protein rich rafts are transported down to the endocytic pathway to reducing late endosomes in mammalian cells (Fivaz *et al.* 2002). Apart from that, GPI-anchoring has been attributed to be a positive signal for internalization into rab5-independent (rab5, a small GTPase located in early endosomes) tubular-vesicular endosomes also responsible for fluid-phase uptake (Sabharanjak *et al.* 2002). Therefore, adoption of such an approach opens up a possibility that GPI anchored PA will undergo its natural cycle upon endocytosis that might improve DNA vaccine potency. As a matter of fact, it is also well documented that CD1 molecules (MHC-I like glycoproteins) survey the endocytic pathway to intersect and bind lipid antigens

(Barral & Brenner 2007). Both human and muroid CD1d molecules (Group 2 CD1 molecules) have been shown to bind to GPI-anchored proteins and present them to a variety of NKT cells that function against infection with pathogens (Schofield *et al.* 1999). Group 2 CD1 (CD1d) molecules have also been shown to control humoral immunity against parasites (Hansen *et al.* 2003), elicit cell-mediated immune responses against tumors (Cui *et al.* 1997), and induce secretion of large amounts of both inflammatory IFN-γ and immunoregulatory interleukin (IL)-4 and IL-10 cytokines (Yoshimoto *et al.* 1994; Wilson *et al.* 1998). Thus, GPI-anchoring of PA might as well aid its presentation in context with CD1 molecules that have the advantage of limited allelic polymorphism yet specialized to bind and present a large repertoire of lipids and glycolipids to T cells.

Based on this rationale we constructed DNA chimera encoding GPI-anchored form of PA utilizing the mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. The GPI signal appears to have been conserved, however, a mammalian GPI signal was specifically attached keeping in mind earlier studies that demonstrate that GPI signals from parasitic protozoa are not recognized by mammalian cells (Moran & Caras 1994). We also designed a chimera in which PA was attached with an N-terminal TPA leader and C-terminal GPI anchor. TPA is expressed by vascular smooth muscle and binds to a specific cellular receptor p63, which occurs in fibroblasts as an intracellular protein associated with the ER (Schweizer *et al.* 1995; Razzaq *et al.* 2003). The TPA leader peptide can therefore, target the expressed antigen directly to the ER thus, obviating the need for the antigen to be processed and translocated to this structure (Ciernik *et al.* 1996). So, we wanted to find out what impact, if any, it would have on DNA vaccine potency as ER is also the site where new CD1d molecules (the only muroid CD1 molecule) are synthesized.

Therefore, with these observations in mind we designed the present study and we successfully illustrated the generation of not only classical MHC II-restricted immunoglobulin (Ig) responses but also non-classical CD1d-restricted Ig responses. Analysis of antibody titers demonstrated successful priming (high IgM titers on first inoculation) and potential IgG titers after the first boost. In vitro cell proliferation studies revealed that there was a clonal expansion of $CD4^+$ $NK1.1^+$ helper T-cell population which rapidly produced IL-4 in response to T cell receptor ligation. Also,

these cells provided direct B-cell help and aided the generation of PA-specific IgG antibodies as indicated by IgG ELISPOT. The chimera pTPA.GPI-PA generated higher PA-specific IL-4 and IgG responses as compared to pGPI-PA. This was attributed to channeling of the antigen to both the classical as well as non-classical pathways of antigen presentation. Effector responses generated by NKT cells isolated from both the groups pGPI-PA and pTPA.GPI-PA in response to GPI-anchored form of PA were found to be completely abrogated by anti-CD1d mAb indicating the involvement of CD1d-mediated restriction. But the group pTPA.GPI-PA also generated MHC-II restricted $CD4^+$ T cell helper responses when presented with non-GPI form of PA which resulted in overall heightened effector responses seen for the group pTPA.GPI-PA as compared to pGPI-PA. Importantly, DNA vaccination mediated the generation of high avidity toxin neutralizing antibodies which efficiently protected animals against anthrax lethal toxin challenge.

MATERIALS AND METHODS

PLASMID DNA PREPARATION. Plasmid DNA was prepared from overnight cultures of transformed DH5α bacteria in Luria Bertani Broth (Amersham) plus 50 μg/ml kanamycin sulfate (Amersham) and processed by using Endo-free Giga kits (Qiagen, Valencia, CA).

PEPTIDES. Peptides (mapped epitopes of PA) used for in vitro stimulation for cells were synthesized commercially (Clover Scientific Pvt. Ltd., India) at >95% purity. Peptide sequences are described in the *Table1*. In addition peptides representing the epitopes of PA attached to a mammalian GPI-anchor sequence *(Table.1)* were also included to determine whether the GPI anchored PA specifically accounted for any of the seen differences in immunological behavior of the proteins. The resulting PA peptide sequences with an additional GPI anchor sequence were also synthesized commercially at > 95% purity.

Table 1. Sequence of the peptides used for in vitro cell stimulation assays.

SEQ ID	SEQUENCE	REFERENCE
PA 64-77I	WSGFIKVKKSDEY	Musson *et al.* 2003
PA 112-127	RLYQIKIQYQRENPTE	Laughlin *et al.* 2007
PA 154-167	PELKQKSSNSRKKR	Musson *et al.* 2003
PA 547-560	ITEFDFNFDQQTSQ	Musson *et al.* 2003
PA 659-672	RYDMLNISSLRQDG	Musson *et al.* 2003
PA 717-730	STNGIKKILIFSKK	Musson *et al.* 2003
GPI-anchor	GTTDAAHPGRSVVPALLPLLAGTLLLLETATAP	Gerber *et al.* 1992

VACCINATION AND LETHAL TOXIN CHALLENGE. Six- to eight-week-old female Swiss albino outbred mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA suspended in phosphate-buffered saline (PBS; 50 μl per hind leg) administered via a

26-gauge, 1-ml hypodermic needle. Following that all the mice received two DNA boosters (100μg each, i.m.) on day 28 and 56. Sera was obtained from blood samples collected from the retro-orbital plexus bleeding prior and post immunization. On different time intervals (12, 14, 16, 18, 20 weeks) post immunization, DNA vaccinated mice were challenged with anthrax lethal toxin (Letx, PA83=50μg and LF=22μg, ≈ 4-5 LD_{50}) injected intravenously via tail vein, and the challenged mice were closely monitored for 21 days.

ELISA DETECTION OF ANTI-PA REACTIVITY IN MOUSE SERUM. The anti-PA reactivity of immunized animal sera was determined by direct ELISA. Briefly, microtiter plates were coated with rPA (10μg/ml) in PBS. Following blocking and washes in PBS-Tween 20 (0.2%), plates were incubated with goat anti-mouse IgG and IgM HRP conjugate (Santacruz Biotechnology) for 1 h at 37°C. After washes in PBS-Tween 20 (0.2%), plates were developed using TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

LETHAL TOXIN NEUTRALIZATION ASSAY. The protective effects of PA-specific Abs were determined using a previously described assay (Staats *et al.* 2007) that measures their capacity to protect the J774A.1 MΦcell line from Letx. Briefly, J774A.1 MΦ ($5x10^4$ MΦ/well) were seeded in 96-well, flat bottom plates. After 12 hrs of incubation, serum dilutions were added together with Letx (187.5ng/ml each of PA and LF, ~4-8 fold toxin dose) and incubated for 4h. Viable MΦ were enumerated after addition of MTT (Sigma-Aldrich). The A_{450} of 100% viable cells was calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

IN VITRO CELL PROLIFERATION ASSAY. After isolation, 4 x 10^7 spleen lymphocytes from naive or DNA vaccinated mice were cultured in the presence of pooled peptides [either GPI-attached PA peptides or non-GPI PA peptides (10μg/ml of each peptide)] with IL-2 (10 U/ml) for a period of 4 days. After that $NK1.1^+TCR\text{-}\alpha/\beta^+$ cells and $CD4^+$ T cells were analyzed by flow cytometry using FACS Vantage™ instrument (Becton Dickinson). All mAbs used in these experiments (mAbs against NK1.1, CD4, and TCR-α/β) were purchased from BD PharMingen. The details of the staining and sorting have been described previously (Nishimura *et al.* 1997). Clonal expansion in the presence of GPI-anchor peptide only *(Table.1)* was subtracted to negate any proliferation response generated specifically against the glycerolipidic region.

ISOLATION OF LYMPHOID CELL SUBSETS BY FACS. Spleen cells were incubated on nylon wool column for 45 min, and the nonadherent cells were used for the isolation of NKT cells and $CD4^+$ T cells by cell sorting using a FACS Vantage™ instrument (Becton Dickinson). All mAbs used in these experiments (mAbs against NK1.1, CD4, and TCR-α/β) were purchased from BD PharMingen. Unless noted otherwise, $CD4^+NK1.1^+TCR\text{-}\alpha/\beta^+$ cells were used as purified NKT cells. The stained cells were isolated using the FACS Vantage™. The purity of the sorted cells was >98%.

IN VITRO IL-4 PRODUCTION. NKT cells and $CD4^+$ T cells (10^5 cells/ml) were incubated with pooled peptides [either GPI-attached peptides or non-GPI peptides (10μg/ml of each peptide)] in the presence of splenocyte APCs (syngeneic splenocytes that were pulsed with peptides and then given mitomycin-C treatment). Antigen induced IL-4 production was determined in the presence and absence of anti-MHC II, anti-MHC I and anti-CD1 monoclonal antibody (Pharmingen, CA, USA). Cell-free culture supernatants harvested at 24 hrs post stimulation and cytokine-specific sandwich ELISA was performed using OptEIA kit for the specific cytokine (BD Pharmingen) according to manufacturer's protocols. IL-4 production in the presence of GPI-anchor peptide only *(Table.1)* was subtracted to negate non-specific stimulation due to the glycerolipidic region only.

B-LYMPHOCYTE PURIFICATION. After isolation, splenocytes were incubated with anti-CD43 and anti-Mac-1 antibody-conjugated microbeads (Miltenyi Biotec). The bead-bound cells (positive fraction) were separated from unbound cells (negative fraction) using an AutoMacs magnetic cell sorter. The enriched cell population was subjected to multiparameter fluorescence-activated cell sorting (FACS) analysis to sort expressing B220, a marker present on cells committed to the B lineage (Li *et al.* 1996). The preparation was 96% $B220^+$, the few contaminants were $CD43^+$/$Mac\text{-}1^+$.

HELPER ASSAYS. B lymphocytes (2.5 x 10^5/ml) were co-cultured in the presence of NKT cells, $CD4^+$ helper T cells or alone in the presence of either, GPI or non-GPI-PA peptides (pooled peptides, 10μg/ml) and IL-2 (10U/ml). Antigen specific IgG production was quantified upon addition of anti-class II and anti-CD1 mAb by ELISPOT. IgG production in the presence of GPI-anchor peptide only *(Table.1)* was subtracted to negate non-specific stimulation due to the glycerolipidic region only. Spots were developed using Alkaline Phosphatase-linked secondary antibody and BCIP/NBT (Sigma-Aldrich) as substrate. Results were expressed as number of SFU (Spot Forming Units) per 10^6 cells.

STATISTICAL ANALYSIS. The experimental data were analyzed by software programs Sigma Plot 8.1 or Excel (Microsoft) and were expressed as mean ± SE. The statistical significance of differences was analyzed by a two-tailed Student's t-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A *P*-value of <0.05 determined was considered statistically significant. Correlation coefficients were determined by linear regression analysis.

RESULTS

Significant serum antibody responses were generated in mice. A correlation between the presence of anti-PA antibody in serum and protection against lethal challenge has been shown previously (Turnbull *et al.* 1988; McBride *et al.* 1998). Therefore, the delivery of DNA vaccine chimeras and generation of measurable humoral responses to PA in the serum of the immunized mice was evaluated by direct ELISA. All pre-immune serum titers of IgG and IgM antibodies against PA were negative. Following i.m. administration, 100% of the mice had strong serum IgM titers *(Table. 2)* which peaked after priming and then declined after administering the first and second booster dose.

Table 2. Anti-PA antibody titers in the serum of DNA immunized animals.

	Antibody end point titers [a], 10^3 ±SD								
	IgM			**IgG**			**TNA**		
Construct	Priming	I[st] Boost	2[nd] Boost	Priming	I[st] Boost	2[nd] Boost	Priming	I[st] Boost	2[nd] Boost
pGPI-PA	0.48±0.1	<0.05	<0.05	25.0±4.0	47.0±6.5[b,c]	79.0±4.1[b,c]	-BD	0.35±0.11[b]	0.51±0.2[b]
pTPA.GPI-PA	0.55±0.2	<0.05	<0.05	13.5±1.5	88.0±5.5[b,c]	100±4.0[b,c]	-BD	0.75±0.23[b]	1.0±0.15[b]
pPA-Nat	0.37±0.2	<0.05	<0.05	3.5±0.5	10.0±2.0	20±2.1	-BD	0.10±0.01	0.15±0.1
Vector Control	BD[d]	-BD	-BD	-BD	-BD	-BD	-BD	-BD	-BD

[a] Mice (n=8-10 per group) were immunized at days 0, 28 and 56; and anti-PA titers were measured in the serum of the immunized mice 14 days after each immunization. Results are expressed as mean titers ±SE for 8-10 mice tested in a group.

[b] Significantly higher ($p<0.001$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c] Significantly higher ($p<0.01$) titers as compared to those developed after priming as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d] Below detection.

Pronounced PA-specific IgG titers were demonstrated by groups that were immunized with chimeras encoding the GPI-anchored form of PA. Highest titers were elicited by the chimera pTPA.GPI-PA (≈100,000), followed closely by the group pGPI-PA (≈79,000) after the second boost. The group immunized with pPA63-native also mounted significant IgG titers as compared to the control mice but the relative titers were lower. Titers after the initial priming were ≥ 3000 and after the first and second booster, titers increased to ≈10,000 and ≈20,000 respectively *(Table. 2)*. Evaluation of Letx neutralization titers revealed that Letx neutralization activity appeared in the serum of animals only after the first booster dose *(Table 2)*. Neutralization titers were approximately 2-3 times higher in the serum of animals immunized with pTPA.GPI-PA and pGPI-PA than the animals vaccinated with the native construct lacking the GPI anchor. Sera from control mice did not neutralize anthrax lethal toxin at all.

$CD4^+$ $NK1.1^+$ helper-T cells were generated upon vaccination with GPI anchored forms of protective antigen. Conventionally, IgG responses require MHC-II restricted recognition of the peptide fragments by $CD4^+$ helper T cells. However, IgG responses to GPI-anchored protein antigens has been found to be regulated in part through CD1d-restricted recognition of the GPI-moiety by $CD4^+$ $NK1.1^+$ helper T cells (Schofield *et al.* 1999). Therefore, we tried to decipher which one of the effector T-cell subset was responsible for B-cell help and hence the generation of immunoglobulin (Ig) responses in vaccinated mice. Flow cytometric analysis revealed that there was a marked increase in both the absolute and relative numbers of $CD4^+$ $NK1.1^+$ helper T-cell population after 5-day culturing in the presence of GPI-attached PA peptides *(Table 3)*. The relative numbers of $CD4^+$ $NK1.1^+$ helper T cells after in vitro proliferation was astonishingly higher in mice immunized with chimera pGPI-PA (75.7%) and pTPA.GPI-PA (71.5%), as compared to those immunized with pPA-native (15%). Importantly, there was also proliferation of $CD4^+$ α/β TCR^+ cells, in vitro, for the group pTPA.GPI-PA. Though this population corresponded only to 18.7%, it proliferated rapidly when it was presented with non-GPI PA peptides giving rise to appreciable number (45.3%). The NKT cell population from this group also proliferated rapidly in the presence of non-GPI PA peptides but the relative number of NKT cells proliferating in response to GPI-

attached PA peptides (71.5 %) was quite higher as compared to those proliferating in response to non-GPI PA peptides (39.7 %). The group immunized with the chimera pPA-Native on the other hand solely gave rise to $CD4^+$ α/β TCR^+ cells in the presence of non-GPI PA peptides only. Overall, chimera pTPA.GPI-PA resulted in proliferation of both $CD4^+$ α/β TCR^+ and $CD4^+$ $NK1.1^+$ helper T-cell population whereas pGPI-PA chimera resulted in clonal expansion of $CD4^+$ $NK1.1^+$ helper T-cell population in majority, in response to GPI-attached PA peptides. Also, the NKT cell population from the group pTPA.GPI-PA responded well against both the forms of PA whereas the one from pGPI-PA responded efficiently only against the GPI-anchored form.

Table 3. Specific phenotype analysis of effector cells by flow cytometry.

	Cell proliferation [a] in the presence of			
	GPI-PA		**Non-GPI-PA**	
Construct	$CD3^+CD4^+$ (%)	$CD4^+NK1.1^+$ (%)	$CD3^+CD4^+$ (%)	$CD4^+NK1.1^+$ (%)
pGPI-PA	5.0	75.7 [b]	10.0	19.5
pTPA.GPI-PA	18.7	71.5 [b]	45.3 [c]	39.7 [c]
pPA-Native	9.6	5.0	55.7	8.6

[a] Splenocytes from naive or DNA vaccinated mice were cultured in the presence of pooled PA-derived peptides [either GPI-attached or non-GPI- PA peptides (10μg/ml of each)] with IL-2 (10 U/ml) over a period of 4 days. Thereafter, $NK1.1^+TCR$- α/β^+ cells and $CD4^+$ T cells were analyzed by flow cytometry.

[b] Significantly higher ($p<0.001$) percentage as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c] Significantly higher ($p<0.05$) percentage as compared to the pGPI-PA group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Combined CD1d- and MHC II-restricted IL-4 responses were generated by chimera pTPA.GPI-PA. *Figure.1.* shows IL-4 production by $CD4^+$ $NK1.1^+$ helper T cells isolated from immunized mice when co-cultured with splenocyte APCs. NKT cell-mediated IL-4 production in the presence of GPI-attached PA peptides was completely abrogated by anti-CD1 mAb in both the groups that received pTPA.GPI-PA and pGPI-PA *(Panel A & E).*

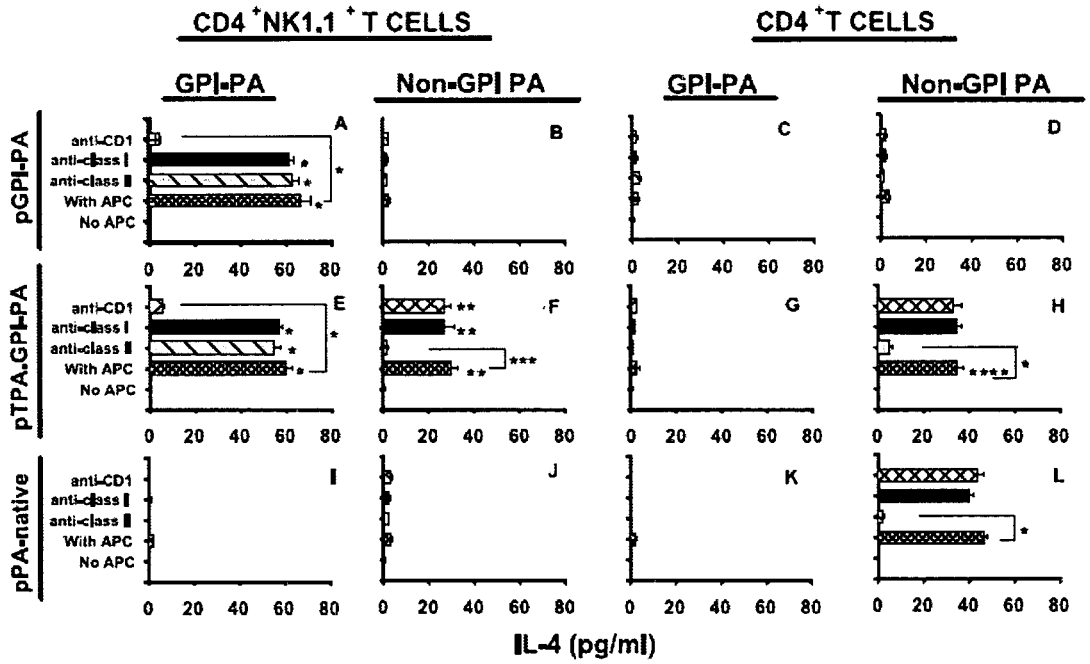


Figure.1. Combined CD1d- and MHC II-restricted IL-4 responses were generated pTPA.GPI-PA chimera. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized to take out the spleens and spleen cells were were used for the isolation of $CD4^+NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. NKT cells and $CD4^+$ helper T cells were cultured in the presence 10μg/ml GPI-PA and non-GPI-PA to determine the cytokine secretion in vitro. Cell-free supernatants were collected 24 h. Each bar represents mean ±SE of IL-4 secreted by NKT cells/$CD4^+$ T cells from 4-6 mice in a group tested in three different experiments. (The error bars represent 95% confidence intervals calculated from the group wise mean concentration. *, **, ***, and ****, Significant difference of the response relative to pPA-Native immunization defined as $p < 0.0001$, $p < 0.001$, $p < 0.01$ and $p < 0.05$, respectively).

These responses on the other hand were completely unaffected by anti-MHC II mAb pointing that GPI-anchored PA was efficiently processed and presented in context with CD1 molecule to NKT cells in these groups. The group immunized with pPA-native did not show any NKT cell mediated IL-4 production in the presence of GPI-attached PA peptides *(Panel I)*. NKT cells isolated from the group

pTPA.GPI-PA also showed NKT cell mediated IL-4 production in the presence of non-GPI PA peptides *(Panel F).* Interestingly, these responses were totally abrogated by anti-MHC II mAb but were completely unaffected by anti-CD1 mAb. Also, evaluation of $CD4^+$ α/β TCR^+ T cell-mediated IL-4 responses indicated that the $CD4^+$ T cells isolated from the group pTPA.GPI-PA and pPA-Native produced IL-4 only when they were presented with non-GPI PA peptides *(Panel H & L).* This lysis was completely abrogated by anti-MHC II mAb and was totally unaffected by anti-CD1 mAb indicating that the antigen was presented in context with MHC-II molecules. On the other hand IL-4 production by $CD4^+$ helper T cells isolated from the group pGPI-PA was insignificant in the presence of both the forms of PA *(Panel C & D).* Overall, the results provided evidence that $CD4^+NK1.1^+$ helper T cells from the group pTPA.GPI-PA mediated CD1d- and MHC-II restricted IL-4 responses against GPI- as well as non-GPI-anchored forms of PA, respectively. $CD4^+$ T cells generated IL-4 responses exclusively against the non-GPI-anchored form of PA. Also, MHC I-restricted responses had no role to play. As a result, the group pTPA.GPI-PA mounted combined CD1d- and MHC II- restricted IL-4 responses whereas the group pGPI-PA and pPA-Native mounted exclusively CD1d-restricted and MHC-II restricted responses, respectively.

CD1d- and MHC II-restricted help to B-cells aided IgG formation. $CD4^+NK1.1^+$ helper T cells provide help to B cells to support Ig formation (Schofield *et al.* 1999). To assess this we co-cultured autologous B lymphocytes with $CD4^+NK1.1^+$ helper T cells in the presence of GPI-attached PA peptides *(Figure.2).* As expected, NKT cells isolated from the group pTPA.GPI-PA and pGPI-PA extended efficient help to B lymphocytes for IgG formation *(Panel A & E, Figure.2).* This response was also completely dependent upon CD1d-restriction of the GPI-attached PA as high SFUs (Spot Forming Units) were obtained only in the absence of anti-CD1d antibody. However, such a response was slightly higher for the group pGPI-PA which developed SFU$\geq$ 600 as compared to the group pTPA.GPI-PA which also displayed a high SFU of $\leq$600 *(Panel A & E).* Importantly, the group pTPA.GPI-PA also displayed MHC II-restricted NKT cell mediated IgG responses against non-GPI PA peptides *(Panel F)* as opposed to only CD1d-restricted responses seen for the group pGPI-PA *(Panel A & B).*

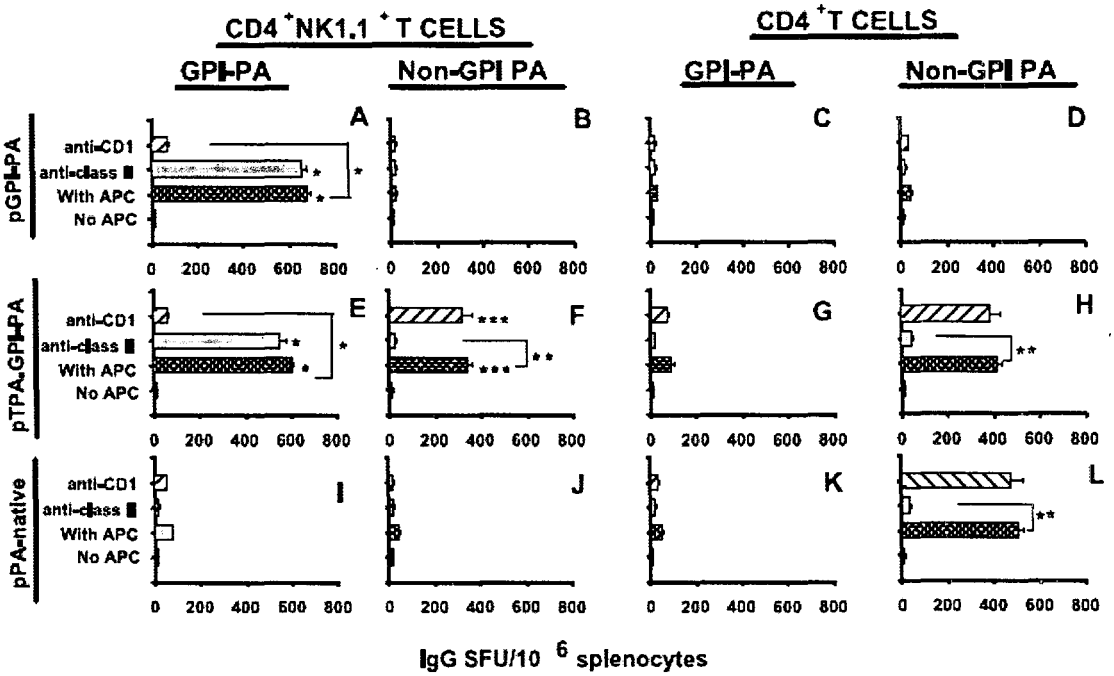


Figure.2. CD1d- and MHC II-restricted help to B-cells aided IgG formation. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized to take out the spleens and spleen cells were were used for the isolation of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. IgG ELISPOT analysis of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ helper T cells in response to GPI-PA and non-GPI-PA day 70 (n=5–6/group). ELISPOT data is expressed as the calculated standard error mean (± SEM) of the Ag-stimulated cells minus un-stimulated cells. Each bar represents number of SFC's obtained for 4-6 mice in a group tested in three different experiments. (The error bars represent 95% confidence intervals calculated from the group wise mean SFC. *, **, ***, and ****, Significant difference of the response relative to pPA-Native immunization defined as p <0.0001, p <0.001, p <0.01and p<0.05, respectively.)

In addition to this, $CD4^+$ T cell-mediated B cell helper responses to non-GPI PA peptides were also evaluated. These results were in corroboration with those obtained for IL-4 production and also detailed the generation of MHC II-restricted IgG responses to non-GPI PA peptides, by both the group pTPA.GPI-PA and pPA-native *(Panel H & L)*. Overall results pointed the development of dual CD1d- and

MHC II- restricted $CD4^{+}NK1.1^{+}$ helper T responses as well as MHC-II restricted $CD4^{+}$ T cell responses for the group pTPA.GPI-PA as opposed to the group pGPI-PA which displayed CD1d-restricted NKT cell helper responses exclusively.

DISCUSSION

Although the currently licensed anthrax vaccine (AVA) generates potential anti-anthrax immunity, it suffers from drawbacks like reactogenicity and long dosage schedule with an additional need for frequent administration of boosters. Clearly, there is reason to explore alternatives. Therefore, based on the hypothesis that GPI-anchored form of PA will improve the DNA vaccination against anthrax, we constructed a DNA chimera encoding the protease-cleaved fragment of protective antigen (PA63) attached to a C-terminal mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. Along with that we constructed another chimera bearing PA63 gene with an N-terminal TPA leader and a C-terminal PLAP-GPI anchor.

In vitro expression of the chimeras in the J774A.1 mouse MΦ-like cells was evaluated to test whether the gene for PA and the signals were being properly recognized in the mammalian cells. Subcellular fractionation and western blotting indicated that the PA protein along with the GPI and TPA signal was being properly recognized by the mammalian cells. The chimeras, pGPI-PA and pTPA.GPI-PA, expressed membrane-anchored form of PA. In addition, the chimera pTPA.GPI-PA also mediated the secretion of PA in the cell culture supernatants.

Earlier studies have underscored the fact that anti-PA antibodies have anti-spore activity and might have a role in impeding the early stages of infection with *B. anthracis* spores (Welkos *et al.* 2001). Also, passive transfer of anti-PA antibodies has been shown to provide protection against anthrax infection in guinea pigs (Little *et al.* 1997). Therefore, to test the ability of GPI anchored PA-expressing chimeras to generate protective humoral responses in vivo, we injected mice with the DNA vaccine chimeras and determined the anti-PA reactivity in the serum of the immunized animals. All vaccinated animals generated potential IgM titers after the first inoculation. These titers declined after the first and second booster suggesting successful priming. Remarkable serum IgG antibody titers were observed after the third immunization for both the constructs encoding GPI-anchored form of PA. These responses were approximately two to three times higher than those generated by pPA-Native chimera indicating that GPI-anchored forms evoked better PA-

specific humoral responses than the non-GPI anchored form of PA. Importantly, electron microscopic studies have shown that spore-associated proteins can be recognized by anti-PA antibodies, and PA-immune serum from several species enhanced the phagocytosis of spores of the virulent Ames and Sterne strains by murine peritoneal macrophages (Welkos *et al.*2001 & 2002; Cote *et al.* 2005). In addition, it has been shown that IgG subclass displays highest affinity towards PA (Little *et al.* 1988). Therefore, development of high end-point titers of IgG antibody upon DNA vaccination gains high merit from anti-spore activity point of view.

Anthrax Letx neutralization titers were also analyzed to assess protection against massive toxemia and septicemia caused by *Bacillus anthracis* tripartite toxin. Sera from the vaccinated animals demonstrated Letx neutralizing activity only after the first booster dose. The mean TNA titers were found to be higher for the groups that received chimeras encoding GPI-anchored form of PA. Peak toxin neutralizing titers appeared at week 14 post immunization. Anti-PA titers of $\geq 10^5$ and neutralization titers $\geq 10^3$ resisted challenge and conferred 100% protection to the animals in the group pTPA.GPI-PA. Earlier studies with guinea pigs (Reuveny *et al.* 2001; Marcus *et al.* 2004) and rabbits (Weiss *et al.* 2006) have shown that anti-PA titers $\geq 10^5$ and ≥ 300, respectively, confer 100% protection. Thus, minimum protective anti-PA antibody titers differ depending upon variables of the host (Fellows *et al.* 2001).

To examine the fine specificity of the T-cell subsets generated upon DNA vaccination we examined the in vitro proliferation of the splenocytes in the presence of GPI-attached- as well as non-GPI-PA peptides which revealed that $CD4^+NK1.1^+$ helper T cells were the main target for activation by GPI-anchored PA. These results were consistent with the seminal study which illustrated that cellular GPI and glycolipids are natural ligands of mouse CD1d, a member of CD1 family of evolutionarily conserved MHC-like molecules (Joyce *et al.* 1998). These CD1d molecules in turn have been shown to directly control the function of $NK1^+$ natural killer T (NKT) cells, a heterogenous subset of T cells displaying a $CD4^+$ or $CD4^-CD8^-$ double negative phenotype, and co-expressing the natural killer cell receptor NK1.1/ NKR P1A and a semi-invariant TCR encoded in mice (Sykes 1990). Interestingly, an appreciable population of NKT cells also resulted from proliferation, in the presence of non-GPI PA peptides, from the group pTPA.GPI-PA

indicating that NKT cells isolated from this group responded well against both GPI as well non-GPI anchored form of PA. However, NKT cells clonally expanded to relatively higher numbers in the presence of GPI-attached PA peptides as compared to non-GPI-associated PA peptides. Results therefore, point that somehow the posttranslational modifications of the expressed antigen in the mammalian systems in vivo affected their ability to activate innate immunity.

Generation of Ig responses against an antigen is as a result of classical MHC-II-dependent "cognate-help" which requires BcR-mediated antigen internalization and presentation of specific epitopes to antigen-specific T cells (Lanzavecchia 1985). Studies however, suggest a direct role for invariant $CD4^+$ NKT cells in regulating CD1d-restricted B cell help which regulates B lymphocyte proliferation and effector functions (Galli *et al.* 2003). Therefore, to establish the restriction requirements of this NKT cell lineage, we evaluated the IL-4 responses. We show here that the recognition of GPI-anchored PA by the NKT cells (from groups pGPI-PA and pTPA.GPI-PA) is MHC-independent and CD1-restricted. However, analysis of similar responses by $CD4^+$ helper T cells isolated from the group pTPA.GPI-PA and pPA-Native indicated that these responses were on account of MHC II-mediated restriction of non-GPI-attached PA. Interestingly, NKT cells from the group pTPA.GPI-PA displayed MHC II-restriction and CD1-independence when presented with the non-GPI PA peptides.

B-cell helper responses generated by $CD4^+NK1.1^+$ helper T cells also paralleled those generated for IL-4 production. The results therefore, gave direct evidence that the NKT cells from the group pGPI-PA extended purely CD1d-restricted help whereas those from the group pTPA.GPI-PA mediated both CD1d- as well as MHC-II restricted B-cell help. In addition, the group pTPA.GPI-PA also gave rise to $CD4^+$ helper T cell population which mediated effector responses that were dependent on classical MHC II restriction of non-GPI anchored PA. All these factors contributed to the heightened IL-4- and B-cell helper-responses obtained for the group pTPA.GPI-PA as compared to those obtained for the group pGPI-PA.

There are reports that have shown that GPI-anchored proteins occur as microdomains in cell membrane of living cells (Friedrichson & Kurzchalia 1998). In this manner the alkyl acyl chain of the GPI-anchored proteins can make contact with the CD1 pocket on the cell surface on account of the unique capacity of CD1

molecules to bind and exchange lipids also on the cell surface (Agea *et al.* 2005). Therefore, generation of predominantly CD1d-restricted NKT cell responses by the group pGPI-PA and pTPA.GPI-PA could probably be as a result of the cell surface recognition of membrane-anchored PA by CD1d molecules. However, the NKT cells and $CD4^+$ helper T cells isolated from the group pTPA.GPI-PA also displayed Ig responses to non-GPI anchored PA that were MHC-II restricted and CD1-independent which suggested that the antigen expressed by this chimera was being channeled to MHC II pathway as well. This could possibly be as a result of deacylation of the expressed GPI-anchored antigen either in ER or upon secretion by endogenous host phospholipases.

TPA is a protein that is expressed by vascular smooth muscle and binds to a specific cellular receptor p63, occurs in fibroblasts as an intracellular protein associated with the ER (Schweizer *et al.* 1995; Razzaq *et al.* 2003). The TPA leader peptide possibly mediated targeting of the expressed antigen directly to the ER which led to the direct loading of the PA on to CD1d molecule. As matter of fact, TPA leader peptide has also been shown to mediate the secretion of the proteins attached to them (Li *et al.* 1999; Delogu *et al.* 2002; Liang *et al.* 2005). This was also seen in our case following in vitro transfection and immunoblot analysis. This secreted (deacylated) protein was channeled to MHC II pathway as indicated by abrogation of effector responses generated by NKT cells and $CD4^+$T helper T cells upon addition anti-MHC II mAb *(Figure. 1& 2).* Overall, there was successful antigen trafficking to both pathways of antigen presentation that is CD1 as well as MHC II in response to GPI-anchored and non-GPI anchored forms of PA, respectively.

Thus, the chimeras encoding the GPI-anchored form of PA successfully mediated the generation of heightened effector responses as compared to those encoding the native form of PA. Hence, the fact that GPI anchored proteins are; internalized via non-clathrin, non-caveolar and dynamin-independent pinocytic pathway to recycling endosomes (Sabharanjak *et al.* 2002); selectively recruited to ER (Udenfriend & Kodukula 1995) and associate with lipid enriched rafts (Fivaz *et al.* 2002); was exploited successfully to target the PA to endocytic pathway as well as the CD1 pathway which mediated efficient effector responses that conferred protection against challenge. To our knowledge there is no published work

demonstrating the development of NKT cell mediated helper responses generated by combined CD1d- and MHC II-restriction of the antigen delivered via i.m DNA vaccination. Responses generated against the GPI-anchored form of PA specifically mediated the maturation and rapid proliferation of $CD4^{+}NK1.1^{+}$ helper T cell subset which rapidly produced IL-4 in response to T cell receptor ligation. These cells were CD1d reactive and upon antigen recognition responded *en masse*.

Therefore, the first and foremost advantage of our vaccination strategy was that we successfully generated CD1 restricted responses whereby the chief player i.e. CD1 molecule shows minimal allelic polymorphism. Also, mouse and human CD1d molecules are widely broadly distributed on most cells of hematopoietic origin, with the highest levels observed on leukocytes such as dendritic cells, B cells or monocytes that will assure constant immune surveillance. Additional help provided by the classical MHC II restricted NKT- and $CD4^{+}$ T-helper cell responses further resulted in the augmentation of useful responses. Therefore, successful targeting to the non-conventional CD1 pathway alongwith the classical MHC II pathway can be devised as a useful strategy for generating heightened humoral immune responses against antigens. We successfully illustrate here the generation of efficient humoral responses against anthrax protective antigen that generated protective immunity against anthrax lethal toxin challenge. Further work can gain a complete understanding regarding the immune pathways involved which can ultimately lead to the design and development of better human vaccines against anthrax to counter emergency.

CHAPTER 6

CORRELATES OF PROTECTIVE IMMUNITY

INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, is a spore forming, gram-positive bacterium. Following inhalation, spores are phagocytosed by alveolar macrophages and transported through the lymphatic channels to hilar and tracheobronchial lymph nodes, where the spores germinate, leading to the multiplication and systemic circulation of vegetative bacilli (Guidi-Rontani *et al.* 1999). Critical to the virulence of *B. anthracis* is the secretion of a tripartite exotoxin consisting of two enzymatically active subunits: lethal factor (LF) and edema factor (EF). These proteins bind to protective antigen (PA), the cell-binding component, to form lethal toxin (Letx) and edema toxin (Etx), respectively (Collier & Young 2006).

The biological activities of Letx and Etx are analogous to those of other A-B toxin systems (Reig & Goot 2006). PA initially binds to a cell surface receptor, including human capillary morphogenesis protein 2 and tumor endothelial marker 8 (Bradley *et al.* 2001, Scobie *et al.* 2003), and undergoes furin-like mediated cleavage of the N-terminal domain. This event yields an amino-terminal 20-kDa fragment and a carboxyl-terminal 63-kDa activated PA63 protein with exposed LF/EF binding domains. The PA63 conformer assembles to form a ring-shaped heptamer with the capacity to bind up to three copies of LF or EF (Klimpel *et al.* 1992; Mogridge *et al.* 2002). At this point the toxin complex is endocytosed. Subsequent acidification of the endosome causes the PA63 heptamer to insert into the membrane, forming a transmembrane channel that traffics LF and EF to the cytosol (Milne *et al.* 1994). LF endopeptidase activity with the MEK family of signal transduction proteins down-regulates both the innate and acquired immune responses by inhibiting cytokine responses, dendritic cell responses, and B- and T-cell immunity (Agrawal *et al.* 2003; Moayeri & Leppla 2004). EF, an adenylate cyclase, incapacitates phagocytes and cytokine pathways through cyclic AMP induction and up-regulates the PA63 receptor on target cells (Hoover *et al.* 1994; O'Brien *et al.* 1985). Given the central role of the toxins in anthrax pathology, the ability to neutralize their effects is of value at all stages of infection.

The credentials of *B. anthracis* as an aerosolized bioterror agent were confirmed by the 2001 postal attacks in the United States, which resulted in five deaths (Jernigan *et al.* 2001). These events underscored the need for post-exposure medical countermeasures that are effective, particularly during middle to advanced stages of infection, when bacteremia and toxemia ensue. Animal studies have previously suggested that early treatment of anthrax is essential since the disease reaches a point when antibiotics are no longer effective due to the accumulation of a lethal level of toxin (Smith & Keppie 1954). In order to counteract the limitations of antibiotics, several attempts have been made to develop new vaccines that can evoke protection against anthrax by targeting PA, due to its central role in pathophysiology (Turnbull 2000).

The currently licensed US vaccine approved for use in humans against infection with *Bacillus anthracis* is Anthrax Vaccine Adsorbed-Biothrax (AVA-Biothrax, formerly identified as AVA and MDPH-PA; BioPort Corporation, Lansing, MI). This vaccine is prepared by adsorbing filtered culture supernatants of the V770-NP1-R strain of *B. anthracis* to aluminum hydroxide (Alhydrogel) and thus is considered a chemical vaccine. Although there is no direct evidence of the efficacy of this vaccine in humans, the low incidence of infection in vaccinated workers who are at risk of exposure to *B. anthracis* spores, such as laboratory workers, veterinarians, and those who work with hides, wool, and bone, suggests that this vaccine is efficacious (Friedlander *et al.* 1999). The efficacy of AVA varies in different animal models. AVA is poorly protective against inhalational anthrax in guinea pigs (Ivins *et al.* 1994) while it is very highly effective in rhesus monkeys (Ivins *et al.* 1996; Pitt *et al.* 1996a). Rabbits are similar to rhesus monkeys in that AVA is highly efficacious against inhalational anthrax (Pitt *et al.* 1996b). At present controlled efficacy studies in humans are not readily available. Clinical studies for anthrax vaccine evaluation now-a-days rely mainly on determination of seroconversion and antibody against PA, yet data on correlation between such titers in human vaccines and protection against exposure are unavailable. Most importantly, the partially defined composition of AVA as well as the requirement for six immunizations followed by annual boosters, underscores the need for the development of new, improved anthrax vaccines.

Given these considerations we report here the development of DNA vaccine against anthrax. For the present study, we developed a mouse model which enables the characterization of circulatory antibodies against PA and also helps determine the interrelationship between the kinetics of anthrax disease onset and memory response mobilization, induced either by lethal toxin challenge or by revaccination. Our data demonstrate that anti-PA titers as well as toxin neutralizing antibody (TNA) titers can act as reliable correlates of protective immunity.

MATERIALS AND METHODS

VACCINATION AND LETHAL TOXIN CHALLENGE. Six- to eight-week-old female Balb/c mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA suspended in phosphate-buffered saline (PBS; 50 μl per hind leg) administered via a 26-gauge, 1-ml hypodermic needle. All mice received the first DNA booster dose 28 days after their initial immunization and received either a second DNA booster (100μg, i.m.) or a s.c. injection of rPA63 (12.5 μg formulation with Incomplete Freunds Adjuvant) 28 days thereafter. Sera were obtained from blood samples collected at 4 wk intervals. For lethal toxin challenge, 50 μg of PA83 and 22μg of LF were injected intravenously via the tail vein, and the mice were closely monitored for 15 days.

ELISA DETECTION OF ANTI-PA REACTIVITY IN PLASMA. Anti-PA reactivity of the sera from immunized mice was determined by direct ELISA. Briefly, microtiter plates were coated with rPA63 (10μg/ml). Following blocking in 5% BSA and washes in PBS-Tween 20 (0.2%), plates were incubated with rabbit anti-mouse anti-PA antibody. Following washes in PBS-Tween 20 (0.2%), anti-rabbit IgG HRP conjugate (Amersham biosciences) for 1 h at 37°C. After another round of washes in PBS-Tween 20 (0.2%), plates were developed using TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

DETECTION OF TOXIN NEUTRALIZING ANTIBODY TITERS (TNA) IN PLASMA. The protective effects of anti-toxin Abs were determined using a previously described assay (Staats *et al.* 2007) that measures their capacity to protect the J774A.1 mouse MΦ-like cell line from Letx. Briefly, J774A.1 MΦ cells (5×10^4 cells/well) were seeded in 96-well, flat bottom plates. After the cells reach 50-70%

confluency, serum dilutions were added together with Letx (187.5ng/ml each of PA and LF) and incubated for 4h. After an additional incubation of 30 min after the addition of MTT (0.5mg/ml) (Sigma-Aldrich), the cells were lysed with acidic isopropanol (0.04-0.1N HCl in 90% isopropanol). The A_{450} of 100% viable cells was calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

DETERMINATION OF AVIDITY OF PA-SPECIFIC IgG ANTIBODIES. Antibody avidity was measured by ELISA using urea as chaotropic agent to dissociate low-affinity antigen-antibody complexes (D' Souza *et al.* 1997). Briefly, serum samples (in twofold dilutions) were incubated for 1 h at 37°C in ELISA plates coated with rPA63. Plates were washed with PBST and soaked with 4 M urea for 10 min. After subsequent washes with PBST, bound IgG was detected with HRP-linked anti-mouse IgG using TMB as substrate. Endpoint titers for each serum sample were determined as described above, in the presence and absence of urea. Results are expressed as an avidity index, calculated as the percentage of residual activity (endpoint titer) using the following formula: (endpoint titer in the presence of urea)/(endpoint titer in the absence of urea) x 100. Positive and negative controls of known antibody titer (calibrated controls) were run in each assay. During the standardization of the assay, several concentrations of urea (0 to 8 M) were tested, and 4 M was selected as optimal; higher urea concentrations completely abrogated antibody binding.

STATISTICAL ANALYSIS. The experimental data were analyzed by software programs Sigma Plot 8.1 or Excel (Microsoft). The statistical significance of differences was analyzed by a two-tailed Students t-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A *P*-value of <0.05 determined was considered statistically significant. Differences in the survival were evaluated using log-rank analysis of the Kaplan-Meier curves. Correlation coefficients were determined by linear regression analysis.

RESULTS

Post-challenge anti-PA and TNA titers, elicited by groups immunized with chimeras bearing TPA and LAMP1 signal, at week 12 and 14 correlated well with survival against lethal toxin challenge. Immunized mice were challenged with a Letx mixture (50μg PA and 22 μg LF, ≈4-5 LD_{50} of Letx) in a total volume of 100 μl via tail vein injection at different time intervals (week 10, 12, 14 and 16) post last immunization. Kaplan-Meier curves for survival of the DNA vaccinated mice against Letx challenge are summarized in *Figure.1*. Control mice (PBS and vector immunized) died after receiving a lethal toxin injection with an average MTTD (Mean Time To Death) around 0.5 ± 0.2 days. Analysis of survival curves indicated that the survival percentage varied at all these time points. Highest survival was elicited by all groups when they were challenged at week 12 and 14. A significant correlation was found between survival percentage and post-challenge anti-PA titers and TNA titers which also displayed their peaks at these two time points ($p<0.001$, *Table 1*).

Statistical differences between the delays in time to death as measured by log-rank statistic, for the groups immunized with TPA and LAMP1 bearing constructs compared to the native construct receiving group were found to be quite significant ($p<0.01$). Time-to-death analysis revealed that DNA vaccination with constructs pTPA-PA63, pPA63-LAMP1and pTPA-PA63-LAMP1 was more protective than the native PA encoding construct. Analysis of the survival percentage displayed by various groups immunized with chimeras bearing the address tags depicted that the group pTPA-PA63-LAMP1 displayed the highest survival frequency of 88% at week 12 and 14 with average MTTD of 8.0±1.5 days. This value was significantly higher than the native group with respect to both survival percentage ($p<0.001$) as well as MTTD ($p<0.01$). Of note, the average anti-PA and TNA titers for this group at these two time points corresponded to $\approx 10^5$ and $\approx 10^3$ (*Table 1*). Other groups that received pTPA-PA63 (Protein boost) and pPA63-LAMP1 (both DNA and protein boost) displayed an average survival percentage of 63-75% with average anti-PA ranging between 65,000-88,000 and TNA titers

between 400-800. The animals that died showed a MTTD of 5.0±1.0 and 4.0±1.5 days for TPA and LAMP1 bearing chimeras, respectively.

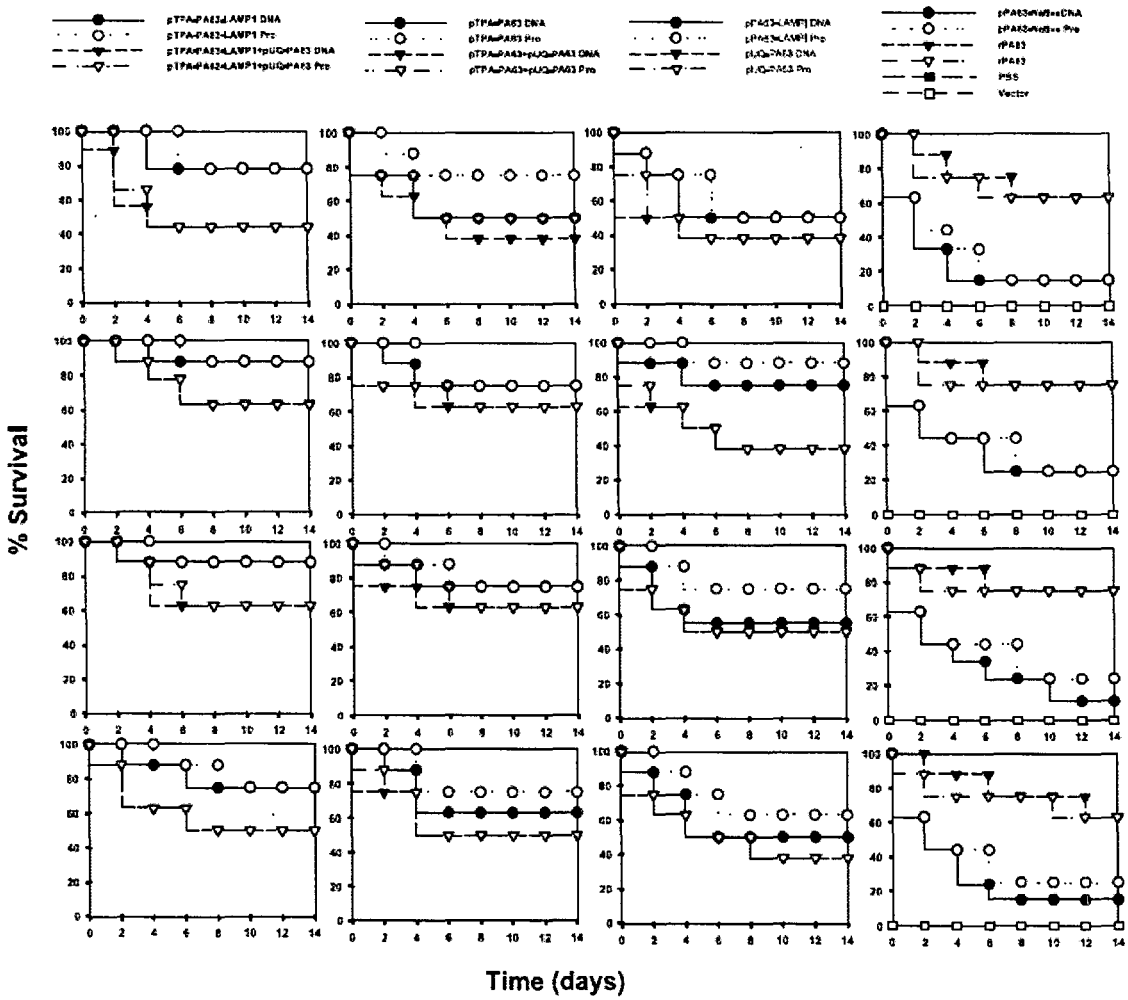


Figure.1. DNA immunization provides efficient protection against Letx challenge. The figure shows Kaplan-Meier curves of survival of the immunized mice challenged with a Letx mixture (50μg PA and 22μg LF) by tail vain (i.v.) injection at different time points post immunization (week 12, 14, 16 and18) and the survival percentage was plotted against the Mean time to death (in days). Challenge was 100% fatal in control mice immunized with vector and PBS. Data shows the percent survival of 9 mice per group compiled by results from three independent experiments.

However, UQ bearing chimeras displayed lowest survival frequency of 38% at these two time points with average MTTD of 5.0±1.0 days. The corresponding value for anti-PA titers and TNA titers for this group was ≈35,000 and ≈250

respectively. Additionally, the groups that received rPA83 and rPA63 also displayed a high survival frequency of 75% with average values of anti-PA and TNA titers of ≥80,000 and ≈800, respectively.

Table 1. Anti-PA and TNA titers post-challenge at different time points.

Group [a]	Total Anti-PA titers [b] (10^3 ± SD)					Letx Neutralizing titers [b] (10^3 ± SD)				
Weeks	10	12 [c]	14 [c]	16	18	10	12 [c]	14 [c]	16	18
pTPA-PA63 Pro	55.5±9.5	87.5±12.5	85±10.5	85±11.1	83±10.3	0.61±0.10	0.82±0.15	0.81±0.2	0.80±0.19	0.79±0.25
pTPA-PA63 DNA	47.0±8.0	78.5±9.2	75.9±10.2	75.5±7.2	72.0±8.0	0.46±0.15	0.70±0.2	0.69±0.1	0.69±0.2	0.67±0.15
pUQ-PA63 Pro	20.5±4.2	35.7±5.7	34.5±5.2	34±5.2	33.5±5.2	0.19±0.02	0.25±0.03	0.24±0.02	0.22±0.03	0.22±0.05
pUQ-PA63 DNA	19.6±4.2	32.6±4.2	32.5±4.2	31.0±2.5	30.0±6.6	0.17±0.02	0.23±0.01	0.22±0.03	0.20±0.02	0.19±0.02
pTPA-PA63-LAMP1 Pro	75.0±4.2	97.5±5.6	100±10.1	98.7±7.2	98.5±9.5	0.76±0.2	1.01±0.3	0.98±0.25	0.97±0.2	0.97±0.35
pTPA-PA63-LAMP1 DNA	69.5±10.5	95.7±10.2	99.5±12.7	97.7±13.5	97.0±9.7	0.69±0.1	1.00±0.1	0.99±0.2	0.99±0.3	0.97±0.2
pPA63-LAMP1 Pro	49.5±6.2	69.5±10.5	65.0±10.0	64.0±6.2	64.0±9.4	0.49±0.09	0.69±0.1	0.69±0.2	0.65±0.1	0.63±0.15
pPA63-LAMP1 DNA	41.0±8.2	57.8±7.6	57.5±4.1	56.0±5.2	55±8.5	0.37±0.1	0.55±0.1	0.55±0.14	0.54±0.1	0.54±0.07
pPA63-NATIVE Pro	10.5±2.1	20.5±3.2	20.5±4.5	19.5±2.2	19±5.7	0.15±0.02	0.19±0.03	0.20±0.06	0.19±0.02	0.15±0.05
pPA63-NATIVE DNA	10.5±3.9	18.8±2.0	19.0±3.0	19.6±5.0	18±5.0	0.15±0.09	0.18±0.02	0.22±0.05	0.20±0.01	0.19±0.04
pTPA-PA63 + UQ-PA63 Pro	25.0±5.5	38.6±7.5	38.5±4.3	35.5±4.2	35.0±5.2	0.29±0.05	0.41±0.09	0.40±0.1	0.39±0.15	0.39±0.1
pTPA-PA63 + UQ-PA63 DNA	22.6±5.2	35.8±6.3	35.5±5.5	34.0±4.2	32.0±4.7	0.25±0.02	0.39±0.05	0.39±0.09	0.37±0.05	0.37±0.9
pTPA-PA63-LAMP1+ UQ-PA63 Pro	30.5±9.2	41.5±9.5	40.5±8.8	40.0±9.5	39.0±9.0	0.39±0.05	0.50±0.1	0.50±0.15	0.49±0.15	0.49±0.13
pTPA-PA63–LAMP1+ UQ-PA63 DNA	27.6±4.2	33.8±5.2	32.5±6.5	32.8±4.4	31.9±2.4	0.31±0.08	0.45±0.12	0.45±0.15	0.41±0.28	0.41±0.14
rPA63	50.5±10.2	80±20.2	80.8±12.6	79.0±15.8	78.5±14.5	0.55±0.09	0.79±0.24	0.79±0.2	0.75±0.20	0.75±0.15
rPA83	53±15.2	88±10.0	87.5±13.7	87.6±15.2	85.5±18.6	0.58±0.1	0.81±0.2	0.80±0.15	0.79±0.21	0.79±0.2

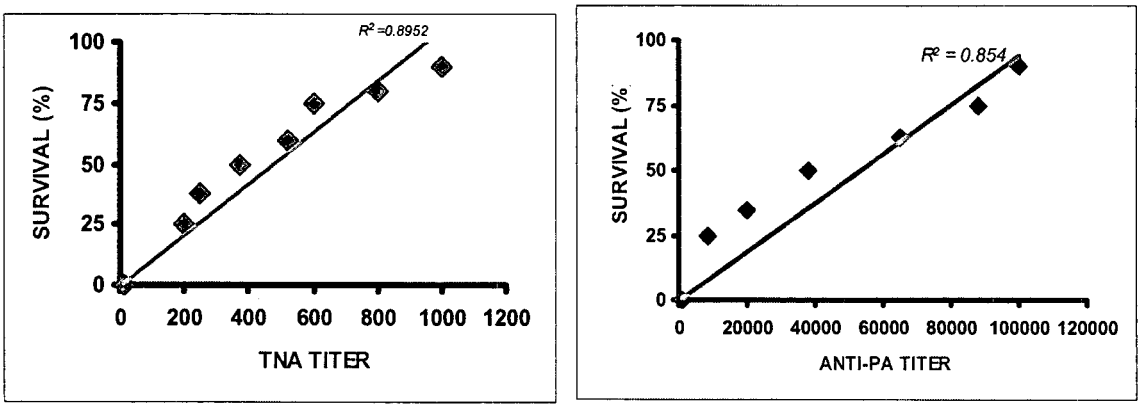
[a] Control mice that received PBS and vector died after challenge.

[b] Mice (n=9 per group) were challenged at different time points post immunization and post-challenge total anti-PA and TNA titers were determined and represented as the reciprocals of dilutions of the serum of the surviving mice, ±SEM.

[c] Significantly higher ($p<0.001$) titers as compared those developed at week 10 as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Rest of the groups displayed a survival frequency of 50-65 % developed anti-PA titers of ≥38,000 and TNA titers ≥350. However, lowest survival percentage of 35% was displayed by the chimera pPA63-Native which developed anti-PA and

TNA titers of ≈20,000 and ≈200, respectively. The results therefore, implied that anti-PA and TNA titers of $\approx 10^5$ and $\approx 10^3$, respectively, provided protection to almost 90% of the animals. Although the chimeras bearing only TPA and LAMP1 signal elicited a high survival percentage, the percentage survival was highest when they were combined together in the chimera pTPA-PA63-LAMP1. Further, when the survival data for all the groups were combined, a significant correlation was found with the protection conferred by the post-challenge anti-PA and TNA titers ($p<0.001$). Correlation analysis *(Figure. 2 A & b)* showed that survival at week 12 and 14 correlated well with the post-challenge anti-PA (r=0.854) and TNA titers (r=0.895). Although significant, MTTD ($p<0.01$, r=0.375) could not be correlated with survival.



(A) **(B)**

Figure 2. Linear regression curve depicting correlation between, A) TNA titer and B) Anti-PA titer and, survival against anthrax lethal toxin challenge. Linear regression was performed using data from experimental cohorts. Calculated r^2 value is depicted on the chart.

Lethal toxin neutralization titers elicited at week 14 and 16 correlated with protection against toxin challenge for groups immunized with GPI bearing chimeras. Immunized mice were challenged with a Letx mixture (50μg PA and 22 μg LF, ≈4-5 LD_{50}) in a total volume of 100 μl via tail vein injection at different time intervals (12, 14, 16, 18 & 20 weeks)post immunization. Toxin challenge results as obtained are summarized in *Table 2.* Control mice (vector immunized) died after

receiving an anthrax lethal toxin injection with a MTTD (Mean Time to Death) around 0.7 ± 0.2 days. All mice immunized with pTPA.GPI-PA resisted toxin challenge and elicited a survival percentage of 100% at all the time intervals post last immunization *(Table. 2).*

Table 2. Correlation of protection against B.anthracis lethal toxin challenge and antiPA-antibody and Letx neutralization titers at different time intervals from last vaccination.

		ANTIBODY TITERS POSTCHALLENGE, 10^3±SD			
Group	Time of challenge [a]	Total Anti-PA Antibody	Letx Neutralizing Antibody	Survival% (No.of survivors/ No. challenged)	MTTD [b] ±SD (Days)
pGPI-PA	12	85.5±5.5	0.65±0.20	100 (9/9)	9.5±0.9[C]
	14	91.4±8.8[C]	0.75±0.10[C]	100 (8/8)	
	16	90.5±7.0[C]	0.70+0.20[C]	100 (7/7)	
	18	80.1±6.5	0.49±0.15	75 (6/8)	9.5±0.5[C]
	20	80.5±7.5	0.45±0.12	75 (5/7)	8.9±0.4
pTPA.GPI-PA	12	120±5.5	1.30±0.50	100 (8/8)	
	14	199±6.0[C]	1.95±0.40[d]	100 (8/8)	
	16	175±8.0[C]	1.70±0.20[d]	100 (5/5)	
	18	155±3.0	1.55±0.15	100 (7/7)	
	20	140±2.0	1.51±0.11	100 (8/8)	
pPA-Native	12	26.0±4.0	0.19±0.05	43 (3/7)	3.6±0.6
	14	41.0±6.5	0.25±0.02	56 (5/9)	5.9±0.2
	16	34.0±3.5	0.24±0.03	56 (5/9)	5.7±0.5
	18	20.0±2.5	0.19±0.03	44 (4/9)	3.6±0.5
	20	19.0±2.5	0.18±0.05	44 (4/9)	3.3±0.6
Vector control	12	-BD [e]	-BD	0 (0/5)	0.70±0.2
	14	-BD	-BD	0 (0/4)	0.70±0.2
	16	-BD	-BD	0 (0/4)	0.70±0.2
	18	-BD	-BD	0 (0/5)	0.70±0.2
	20	-BD	-BD	0 (0/6)	0.70±0.2

[a] The immunized mice were challenged at 12, 14, 16, 18, 20 weeks post last immunization with anthrax lethal toxin mixture of PA (50 μg/mouse) and LF (22 μg/mouse) injected i.v. through tail vein and monitored for 15 days.

[b] The mean time to death (MTTD) was determined by determining the average time to death for each group, excluding the survivors.

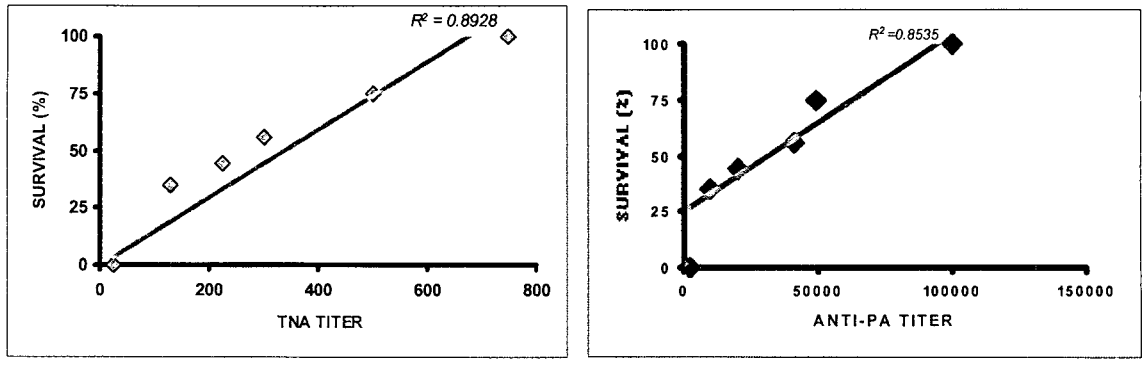
[c] Significantly higher ($p<0.01$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d] Significantly higher ($p<0.001$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[e] BD, below detection.

The average neutralization titers for this group were $\geq 10^3$ and anti-PA titers were $\geq 10^5$. On the other hand, the group pGPI-PA showed a 100% survival till week 20 post immunization with average neutralization titers for protection ranging between 600-800. Letx neutralization titers below this range (400-500) conferred protection to 75-80% of the mice and increased MTTD to 8.5-10.4 days. Results also depicted that both anti-PA and Letx neutralization titers leveled off at titers about $\geq 10^5$ and $\geq 10^3$ respectively, for the group pTPA.GPI-PA; and ≥80,000 and 450 respectively, for the group pGPI-PA 6 weeks after the second booster dose. For the group pPA-Native, neutralization titers ranging from 100-300 conferred protection to 45-55% of the mice with an average MTTD ranging from 3.3 to 5.9 days.

Statistical differences between the delays in time-to-death as measured by log rank statistic for the groups immunized with GPI-bearing chimeras compared to the vector control group showed that the differences were quite significant ($p<0.01$). Analysis of post-challenge TNA titers (r=0.89) and anti-PA titers (r=0.85) indicated that both these factors correlated well survival at week 14 and 16 *(Figure.3 A & B)*. The results therefore, suggested that mice immunized with DNA vaccine chimeras bearing GPI-anchor developed significant Letx neutralization titers which protected the mice against anthrax lethal toxin challenge. Importantly, DNA immunization alone with the chimera pTPA.GPI-PA provided 100% protection to animals till 140 days post priming.



(A) **(B)**

Figure 3. Linear regression curve depicting correlation between, A) Post-challenge TNA titer and B) Post-challenge Anti-PA titer and, survival against anthrax lethal toxin challenge. Linear regression was performed using data from experimental cohorts. Calculated r^2 value is depicted on the chart.

Kinetics and characterization of immunological memory post-immunization and challenge. Avidity of the antibodies generated against PA was determined by urea-based ELISA described in the materials and methods. Avidity index (AI) of the antibodies generated was ≤ 40 in all animals 2 weeks after the second boost *(Table 3).*

Table 3. Avidity indices of anti-PA antibodies in the serum of immunized mice.

Construct	Antibody avidity index [a]		
	Week 12	Week 14 (Challenge)	Week 16 (Post challenge)
pTPA-PA63 Pro	62	71	75 [b), d)]
pTPA-PA63 DNA	45	49	63 [d)]
pUQ-PA63 Pro	30	36	45
pUQ-PA63 DNA	25	34	40
pTPA-PA63 -LAMP1 Pro	80	85	92 [b), d)]
pTPA-PA63 -LAMP1 DNA	75	81	87 [b), d)]
pPA63-LAMP1 Pro	73	77	85 [c)]
pPA63-LAMP1 DNA	55	61	67
pPA63-NATIVE Pro	41	45	58
pPA63-NATIVE DNA	39	44	50
pTPA-PA63 +UQ-PA63 Pro	43	46	53
pTPA-PA63 +UQ-PA63 DNA	36	39	47
pTPA-PA63 -LAMP1+ UQ-PA63 Pro	57	67	72
pTPA-PA63 -LAMP1+ UQ-PA63 DNA	45	49	54
pGPI-PA	33	62 [b), d)]	78 [b), d)]
pTPA.GPI-PA	39	79 [b), d)]	87 [b), d)]
pPA-Native	23	34	53
rPA63	75	78	82 [b)]
rPA83	80	86	75 [c)]
PBS	- [e)]	-	-
VECTOR	-	-	-

[a)] Avidity index= (endpoint titer in the presence of urea)/ (endpoint titer in the absence of urea)) x 100.
[b)] Significantly higher ($p<0.001$) avidity index as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c)] Significantly higher ($p<0.01$) avidity index as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d)] Significantly higher ($p<0.01$) avidity index as compared as compared to those groups that received their combinations with pUQ-PA63 chimera as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[e)] No PA-specific antibody was detected from the serum of the control mice. These mice died after toxin challenge.

Animals inoculated with pPA-Native chimera had the lowest avidity index of 23. Following anthrax lethal toxin challenge at week 14, intermediate to high avidity antibodies were generated by the immunized mice. Group immunized with pTPA.GPI-PA and pGPI-PA showed higher avidity indices of 79 and 62 respectively, whereas the group pPA-native showed a significantly lower AI value of 34. Two weeks post challenge (at week 16), a further increase in AI was observed for all the groups *(Table 3)* with groups pTPA.GPI-PA and pGPI-PA showing avidities as high as 87 and 78 respectively, as opposed to an AI=53 for the pPA-Native group. Similarly all the groups that received constructs bearing TPA, LAMP1 address tags showed high avidities (AI ranging from 65 to 90). Their combinations with UQ bearing chimera also elicited increased avidities (AI≥45) indicating affinity maturation. Overall the results paralleled the antibody measurement experiments and suggested that partial maturation does take place after a regimen of immunization, however, robust memory responses were induced post-challenge at week 14 indicating a challenge engendered anamnestic immune response.

Evaluation of the protective potential of anti-PA antibodies by passive transfer. To further evaluate the protective value of the antibodies generated by DNA immunization, homologous passive-transfer experiments were performed. Various dilutions of hyperimmune serum (volumes of 0.5 ml with antibody titer ranging from 10,000 to 100,000) obtained from DNA immunized mice (2 weeks after a schedule of two immunizations with the DNA vaccine followed by a rPA63 protein booster) were introduced i.p. into mice. One day after serum transfer, the animals were challenged. As in the previous experiments, correlation between circulatory antibody titers and protection was evaluated *(Figure. 4)*. Approximately 78% (7 out of 9) of the mice with a TNA titer ranging from 450-600 were fully protected whereas the mice with TNA titer $\approx 10^3$ resisted lethal toxin challenge.

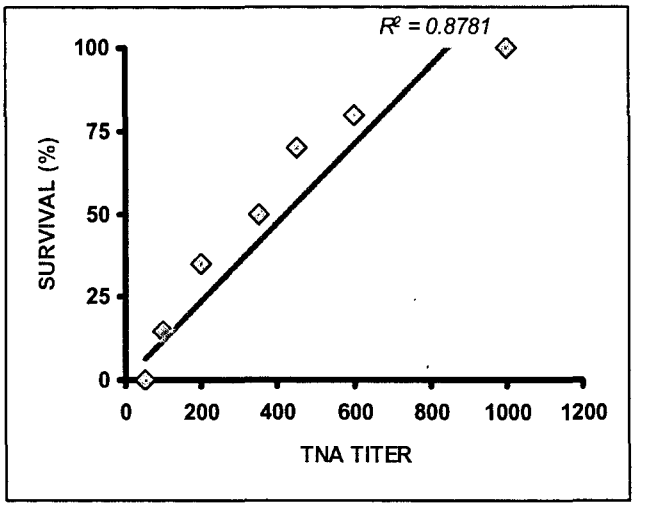


Figure 4. Passive immunization with PA hyper-immune: Linear regression curve depicting correlation between TNA titer and survival against anthrax lethal toxin challenge. Linear regression was performed using data from experimental cohorts. Calculated r^2 value is depicted on the chart.

DISCUSSION

For any vaccine to be licensed for human use, it becomes a necessity to define its appropriate correlates of protection. Gathered information, gained over periods of research on anthrax vaccines, indicates that antibodies specific for protective antigen (PA) confer protection against live infection (Turnbull *et al.* 1988; Little *et al.* 1977; McBride *et al.* 1998; Pitt *et al.* 2001; Fellows *et al.* 2001). Studies have pointed out that anti-PA antibodies induced by vaccines have anti-spore activity and probably have a role in impeding early stages of infection with *Bacillus anthracis* (Welkos *et al.* 2001 & 2002). In addition toxin neutralizing antibodies against PA have been shown to inhibit spore germination (Cote *et al.* 2005). These findings are also supported by earlier studies demonstrating the protective activity of monoclonal antibodies (MAb) specific for PA (Little *et al.* 1988). These MAb's retarded germination in vitro, and enhanced the phagocytic and sporicidal activities of macrophages.

Further, studies in which various vaccines were evaluated for their protective potential have indicated that elevation in the level of PA-specific antibodies was accompanied by increased survival. This was found in cell-free vaccines when various vaccine formulations were compared (Fowler *et al.* 1999; Ivins *et al.* 1992; Turbull *et al.* 1986), in attenuated live vaccines where production levels of PA were altered (Barnard & Friedlander 1999; Cohen *et al.* 2000), and also in passive immunization using PA hyperimmune serum (Little *et al.* 1997). In these cases, serum levels of lethal toxin neutralizing antibody (Reuveny *et al.* 2001) and total serum anti-PA IgG (Pitt *et al.* 2001; Little *et al.* 2004) have significantly correlated with protection against challenge with virulent spores. However, the reported studies are not necessarily in agreement as to the level of antibody required to protect animals against anthrax challenge. Correlations have proven informative within studies, but no consensus requirement for minimum levels of anti-PA serum or toxin neutralizing antibody is emerging, even within a single species.

The principal animal models used in laboratory investigations of experimental anthrax have been mice, rats, guinea pigs, rabbit and rhesus monkeys. Rhesus monkeys are considered as the best model of inhalational anthrax in humans

(Berdjis *et al.* 1962; Gleiser 1967; Fritz *et al.* 1995). The disease induced by respiratory exposure to spores is a rapidly fatal illness, death occurring between the second and seventh days post-exposure. However, rhesus monkeys inoculated with two doses of the licensed vaccine, AVA, were protected against a lethal aerosol challenge of anthrax spores for up to 2 years (Ivins *et al.* 1996).

Studies in primate model necessitate the requirement of large numbers of monkeys to produce statistically significant data for the evaluation of an in vitro correlate of immunity. All this makes the use of rodents or lagomorphs more desirable for large scale studies. However, mouse strains differ significantly in their innate susceptibility to lethal infection by both a fully virulent strain and the non-encapsulated Sterne vaccine strain (Welkos *et al.* 1986 & 1989). Mice can be protected against attenuated, un-encapsulated anthrax strains with various vaccines but not against virulent organisms (Welkos *et al.* 1988). Of note, anthrax is primarily a systemic disease resulting from the release of anthrax toxin in peripheral lymph nodes and in the general circulation (Dixon *et al.* 1999; Mock & Fouet 2001). Therefore, development of effective cellular and humoral immunity in the systemic compartment is of immense importance. Keeping this in mind, we monitored the ability of DNA chimeras to confer protective immunity in mice following lethal toxin challenge and tried to find out the minimal anti-PA and TNA titers that were protective against toxemia.

Importantly, immunization studies with AVA (US anthrax vaccine) have demonstrated a decreasing serum anti-PA antibody titer in humans over time (Pittman *et al.* 2002). Also a gradual decline in antibody titer over time, a parameter that is currently being used to determine the immunological status after vaccination, argues for periodic booster inoculations to maintain an appreciable titer. Thus, many variables are evaluated to predict the correlates of protective immunity, for e.g., persistence of circulating antibody post-immunization, antibody titers generated in surviving mice post-challenge and the time at which peak titers are attained or the time elapsed before the titers return to baseline.

To this end, we assessed the anti-PA antibody response, in terms of total anti-PA and TNA titers prevailing in vaccinated animals post-challenge that aided protection. Results detailed that anti-PA and TNA titers were maintained until week 18 *(Table.1)*. Comparison of the antibody titers between various groups indicated

that the chimeras bearing the address tags TPA, LAMP1 or both, mounted pronounced anti-PA and TNA titers. Changes in the survival of the immunized mice with regard to anti-PA and TNA titers revealed that titers at week 12 and 14 were quite significant ($p<0.001$, *Table1*). Anti-PA titers of $\approx 10^5$ and TNA titers of $\approx 10^3$ provided protection to ≈90% of the vaccinated animals. However, the mean TNA titers for the groups that received chimeras encoding GPI-anchored form of PA peaked at week 14 and 16 post immunization. Anti-PA titers of $\geq 10^5$ and neutralization titers $\geq 10^3$ resisted challenge and conferred 100% protection to the animals in the group pTPA.GPI-PA. In certain studies with guinea pigs (Reuveny *et al.* 2001; Marcus *et al.* 2004) and rabbits (Weiss *et al.* 2006) it has been shown that anti-PA titers of ≥300 and $>10^5$ respectively, confer 100% protection. Thus far, the minimum protective anti-PA antibody titers differ depending upon variables of the host (Fellows *et al.* 2001). Earlier observations have also pointed that disease pathogenesis or intrinsic antibody titers differ with respect to host (Reuveny *et al.* 2001; Weiss *et al.* 2006; Pittmann *et al.* 2002; Little *et al.* 2004) and thus, contribute to the variable predictors of protective immunity.

Further analysis demonstrated that quantitative anti-PA and TNA titers at week 12 14, in addition to displaying their peaks at these two time points, were significant predictors of survival for the groups immunized with chimeras bearing TPA and LAMP1 address tags. Correlation analysis showed that survival at week 12 and 14 correlated well with the post-challenge anti-PA (r=0.854) and TNA titers (r=0.895) *(Figure. 2A & B).* Although significant, the mean-time-to-death ($p<0.001$, r=0.375) could not be correlated with survival at these two time points. Similarly, survival at week 14 and 16 correlated well with the anti-PA (r=0.85) and TNA titers (r≈0.89) developed at these two time points.

In previous studies with AVA immunized NZW rabbits, anti-PA and TNA titers at week 6, 8 and 10 were found to be significant predictors of immunity (Little *et al.* 2004 & 2006). Likewise, another study with guinea pigs indicated that the anti-PA and TNA titers at the time of challenge (week 6) were of predictive value in evaluating survival (Reuveny *et al.* 2001). Further it was reported that survival of guinea pigs that had been challenged 3 months after being inoculated with two doses of rPA (either 50, 5, or 0.5 μg per dose) formulated with Alhydrogel at 0 and 4 weeks was not dose dependent (Pitt *et al.* 1996b). However, another study argued

against multiple inoculations and instead favored a single inoculation of vaccine (Reuveny *et al.* 2001). They demonstrated that their TNA assay could serve as an effective correlate of protection for guinea pigs that were challenged intradermally with spores of the Vollum strain of *B. anthracis* after a single subcutaneous inoculation of rPA (either 25, 12.5, 6.3, 3.1, 1.6, or 0.8μg) adsorbed to Alhydrogel. Possible factors contributing to differences between earlier studies and ours may be the form of antigen (DNA vs. protein) or else the animal model (mice vs. rabbits and guinea pigs). Moreover, comparison of antibody levels with those obtained in the previous studies with AVA (Pitt *et al.* 2001) is problematic because of several factors: (i) the possible adjuvant effect of LF and other undefined bacterial and medium proteins that are present in AVA; (ii) the Alhydrogel concentration in AVA; and (iii) varying and unknown amounts of PA in each AVA vaccine lot preparation.

Further support for the utilization of neutralizing antibody titer as a valid marker for protection was provided by passive-immunization experiment, in which anti-PA antibodies were transferred to naïve mice. Results pointed that approximately 78% (7 out of 9) of the mice with a TNA titer ranging from 450-600 were fully protected whereas the mice with TNA titer $\geq 10^3$ resisted lethal toxin challenge. These results were in clear corroboration with those obtained from active immunization experiments. The importance of antibodies to PA in passive protection has also been shown earlier by hyperimmune serum transfer obtained from guinea pigs after active immunization with recombinant PA (AVA) (Little *et al.* 1997). However, evaluation of correlates of protection indicated that full protection correlated with titers of at least 300 (Reuveny *et al.* 2001). Thus, the quantitative TNA titers were found to be significant predictors of immunity and underlines the importance of the production of a critical protective antibody mass in due time.

Investigation of memory responses in terms of affinity maturation of antibodies (antibody avidity) upon challenge indicated that a challenge engendered amanestic immune response was mounted. There was progressive increase in the avidity of the antibodies on subsequent immunizations. However, antibodies of highest avidity were generated following anthrax lethal toxin challenge at week 14. Also, there was a further rise in avidity at week 16 (two weeks post challenge). The result of this set of experiments substantiates previous observations and underscores the delicate balance between the time required to generate functional antibody-

producing cells from the resting precursor memory cells and the time course of disease progression. This balance between the onset of protection and time to death could be different in different animal models. All together, all these factors can affect the protective efficacy of the memory response.

Although the role of humoral immune response to PA as a surrogate marker for protection has been exemplified, protection mediated by PA has also been shown to be T-cell dependent (Williamson *et al.* 1990 & 1999). Also, a recent study has suggested the role of IFN-γ producing $CD4^+$ T lymphocytes in protection against anthrax (Glomski *et al.* 2007). Therefore, the central role of adaptive cellular immunity cannot be precluded. However, a clear correlation between TNA titers/anti-PA titers and protective immunity demonstrated in our study indicated that both these parameters can serve as reliable correlates of protection. Also, our study investigated for the first time a serological marker of DNA-vaccine induced immunity and defined the antibody titers that provide protection in a mouse model against anthrax lethal toxin challenge. These results can be of immense value in determining the immune status of DNA vaccinated animals and can be instrumental in assessing the efficiencies of various novel vaccine formulations.

SUMMARY AND CONCLUSIONS

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Based on the hypothesis that immune outcome can be influenced by the form of antigen administered, we mediated the targeting of 63kDa fragment of protective antigen (PA) of *Bacillus anthracis* to various sub-cellular locations by DNA chimeras bearing a set of signal sequences. These targeting signals namely, LAMP1 (Lysosome Associated Membrane Protein 1), TPA (Tissue Plasminogen activator), Ubiquitin and GPI (Glycosyl Phosphatidyl Inositol anchor) encoded various forms of PA (protective antigen) viz. lysosomal, secreted, cytosolic and membrane-anchored forms, respectively. Immune outcome detailed that the heightening of immune responses appeared to be rooted in the differences in the targeting signals attached and their ability to traffic PA63 to various antigen-processing pathways. LAMP1 and TPA signals mediated targeting of PA to MHC class II pathway. Ubiquitin specifically pumped PA to MHC class I pathway. On the contrary, a C-terminal GPI anchor signal mediated targeting to CD1 pathway. However, when combined with an N-terminal TPA leader, GPI anchor mediated the generation of MHC class II as well as CD1d-restricted immune responses.

Differential processing in these compartments gave rise to both qualitative and quantitative differences in the generation of peptides arising from these compartments that ultimately lead to the variations in the immune response generated by different groups. A more striking result was evident in our immunization setting, i.e. the protective effect was maximal when the two signal sequences, TPA and LAMP1/ or else TPA and GPI, were combined together as N- and C-terminal tags. Thus, the immune outcome showed a greater dependence on targeting of the antigen to appropriate antigen processing pathway (s) rather than on prime-boost regimen.

Furthermore, the membrane-anchored (pGPI-PA) and secreted-membrane anchored (pTPA.GPI-PA) form of PA mediated successful antigen trafficking to both pathways of antigen presentation i.e. CD1 as well as MHC class II in response to GPI-anchored and non-GPI anchored forms of PA, respectively. Overall, successful targeting of immunologically relevant antigen to the conventional (MHC class I & MHC class II) and non-conventional (CD1) pathways of antigen

processing, was devised as a useful strategy for generating effective cellular and humoral immunity in the systemic compartment that helped generate protective immunity against anthrax lethal toxin challenge. Important findings of the thesis work are mentioned below in a sequential order:

- The structural gene for PA63 (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using sequence-primers and cloned in DNA plasmids bearing the address tags. The nucleotide sequences of clones bearing the address tags were submitted to the Gene Bank, pPA63-Native: (GenBank accession no. EU249810), pTPA-PA63 (GenBank accession no. EU249808), pUQ-PA63 (GenBank accession no. EU249809), pPA63-LAMP1 (GenBank accession no. EU249806), pTPA-PA63-LAMP1 (GenBank accession no. EU249807).
- The authenticity of the DNA chimeras and the ability of the address tags to target the encoded Ag to desired subcellular locations was confirmed by transfection analysis followed by subcellular fractionation and immunoblotting. Results indicated that the PA63 protein along with the targeting signals was being properly recognized by the mammalian cells.
- The structural gene for protective antigen (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using sequence-primers and cloned in pQE30 vector (T7 expression system).
- Further, recombinant PA63 was expressed in *E.coli* M15 cells. An attempt was made to purify recombinant PA under denaturing conditions using metal affinity chromatography. Using this approach, 3 mg of protective antigen was purified to >95% from 1 litre culture.
- Analysis of the functional and biological activity of the purified recombinant PA63 expressed in *E. coli* indicated that the protein was biologically active and mediated its function as the receptor binding moiety of anthrax toxin complex in a manner similar to that displayed by full-length PA83 protein.
- DNA chimeras induced a strong and long lasting systemic anti-PA antibody response following inoculation in mice. No anti-PA reactivity was seen in the serum of mice immunized with PBS and corresponding vector.
- Following i.m. administration, 100% of the mice developed significant IgG1 antibodies after the first booster dose. Significantly higher anti-PA IgG1 titers

(≈40,000) were observed following immunization with the construct encoding pTPA-PA63-LAMP1 (Protein boost) as compared to the native construct immunized group and other groups immunized with constructs bearing the address tags.

- Evaluation of the anti-PA IgA end point titers displayed no significant differences between various groups however; titers $\geq 10^3$ were maintained for over a period of 22 weeks.
- Furthermore, evaluation of the titers of complement fixing antibody i.e. IgG2a, depicted that titers of $\geq 10^4$ developed in the group immunized with pTPA-PA63, pTPA-PA63-LAMP1 and pUQ-PA63 after the second boost. For rest of the groups the titers ranged between 10^3 and 10^4.
- A similar trend was also seen for anti-PA IgG2b antibody which also displayed titers ranging between 10^3 and 10^4.
- For all the groups that received chimeras bearing TPA, LAMP1 address tags, the IgG1 titers were approximately 1.0 log higher than the IgG2a responses which clearly indicated a TH2 bias. On the contrary, the chimera pUQ-PA63 displayed a TH1 bias on account of a lower IgG1: IgG2a ratio.
- Cytokine measurement experiments depicted that TPA and LAMP1 signals biased the response towards TH2 type however; the bias was much more pronounced when these two signals were combined together as N- and C-terminal address tags. Contrarily, immunization with UQ bearing construct, alone or in combination, resulted in a preferential bias towards a TH1 type immune response.
- TPA, LAMP1 bearing chimeras generated an overall higher count of IL-4 secreting cells as compared to IFN-γ secreting cells. However, their combinations with pUQ-PA63 elicited an overall lower frequency of IL-4 SFUs as compared to when they were administered alone.
- Chimeras bearing LAMP1 and TPA address tags favored efficient recall B-cell responses upon re-stimulation with PA in vitro.
- Almost all the groups mounted strong proliferative responses which indicated successful clonal expansion of PA-specific splenic cells on re-stimulation in vitro.

- $CD4^+$ T-cell cytotoxicity was displayed by the groups immunized with chimeras bearing TPA, LAMP1 address tags whereas predominantly $CD8^+$ T-cell cytotoxicity was displayed by groups receiving UQ-bearing chimera.
- Pre-challenge TNA titers were significantly higher in the serum of animals immunized with chimeras bearing both the TPA and LAMP1 address tags than the animals immunized with the chimeras bearing them independently or lacking them.
- Differential processing in various cellular compartments gave rise to both qualitative and quantitative differences in the generation of peptides that probably explains why we observed differences in the magnitude and type of immune responses generated by the chimeras bearing TPA and LAMP1 signals although both of them targeted the antigen to endosomal pathway.
- Apart from this, the combinations that received protein boosters also mediated heightened responses as compared to the groups that received a DNA booster.
- However, the results also pointed that immune outcome showed a greater dependence on targeting of the antigen to appropriate cellular compartment (s) rather than on prime-boost regimen.
- Pronounced PA-specific IgG titers were demonstrated by groups that were immunized with chimeras encoding the GPI-anchored form of PA.
- Evaluation of Letx neutralization titers revealed that Letx neutralization activity appeared in the serum of animals only after the first booster dose. Neutralization titers were approximately 2-3 times higher in the serum of animals immunized with pTPA.GPI-PA and pGPI-PA than the animals vaccinated with the native construct lacking the GPI anchor. Sera from control mice did not neutralize anthrax lethal toxin at all.
- Flow cytometric analysis revealed that there was a marked increase in both the absolute and relative numbers of $CD4^+$ $NK1.1^+$ helper T-cell population after 5-day culturing of spleen lymphocytes from DNA vaccinated mice in the presence of GPI-attached PA peptides.
- Chimera pTPA.GPI-PA resulted in proliferation of both $CD4^+$ α/β TCR^+ and $CD4^+$ $NK1.1^+$ helper T-cell population whereas pGPI-PA chimera resulted in clonal expansion of $CD4^+$ $NK1.1^+$ helper T-cell population in majority, in response to GPI-attached PA peptides. Also, the NKT cell population from the

group pTPA.GPI-PA responded well against both the forms of PA whereas the one from pGPI-PA responded efficiently only against the GPI-anchored form.

- The results provided evidence that $CD4^+NK1.1^+$ helper T cells from the group pTPA.GPI-PA mediated CD1d- and MHC-II restricted IL-4 responses against GPI- as well as non-GPI-anchored forms of PA, respectively.
- $CD4^+$ T cells generated IL-4 responses exclusively against the non-GPI-anchored form of PA.
- MHC I-restricted responses had no role to play.
- As a result, the group pTPA.GPI-PA mounted combined CD1d- and MHC II-restricted IL-4 responses whereas the group pGPI-PA and pPA-Native mounted exclusively CD1d-restricted and MHC-II restricted responses, respectively.
- Results pointed the development of dual CD1d- and MHC II- restricted $CD4^+NK1.1^+$ helper T responses as well as MHC-II restricted $CD4^+$ T cell responses for the group pTPA.GPI-PA as opposed to the group pGPI-PA which displayed CD1d-restricted NKT cell helper responses exclusively.
- CD1d- and MHC II-restricted help to B-cells aided IgG formation.
- Results pointed that the TPA leader peptide possibly mediated the targeting of the expressed antigen directly to the ER which led to loading of the antigen on to CD1d molecule. These responses were totally abrogated upon the addition of CD1 antibody.
- TPA leader peptide also mediated the secretion of the encoded protein antigen (as shown by sub-cellular fractionation and western blotting) which was possibly deacylated by host endogenous phospholipases.
- This secreted (deacylated) protein was channeled to MHC II pathway as indicated by abrogation of effector responses generated by NKT cells and $CD4^+$T helper T cells upon addition anti-MHC II mAb.
- Overall, there was successful antigen trafficking to both pathways of antigen presentation that is CD1 as well as MHC II in response to GPI-anchored and non-GPI anchored forms of PA, respectively.
- Post-challenge anti-PA ($r=0.854$) and TNA titers ($r=0.895$), elicited by groups immunized with chimeras bearing TPA and LAMP1 signal, at week 12 and 14 correlated well with survival against lethal toxin challenge.

- Although significant, MTTD (p<0.01, r=0.375) could not be correlated with survival.
- Lethal toxin neutralization titers (r=0.89) and anti-PA titers (r=0.85) elicited at week 14 and 16 correlated with protection against toxin challenge for groups immunized with GPI bearing chimeras.
- High avidity toxin neutralizing antibodies were generated following immunization with chimeras bearing the address tags which indicated partial affinity maturation after a regimen of immunization.
- However, robust memory responses were induced post-challenge at week 14 indicating a challenge engendered anamnestic immune response.
- Anti-PA titers of $\approx 10^5$ and TNA titers of $\approx 10^3$ provided protection to ≈90% of the vaccinated animals.
- Passive antibody (from DNA immunized mice) transfer experiment suggested that approximately 78% (7 out of 9) of the mice with a TNA titer ranging from 450-600 were fully protected whereas 100% of the mice with TNA titer $\approx 10^3$ resisted lethal toxin challenge. These results were in clear corroboration with those obtained from active immunization experiments.

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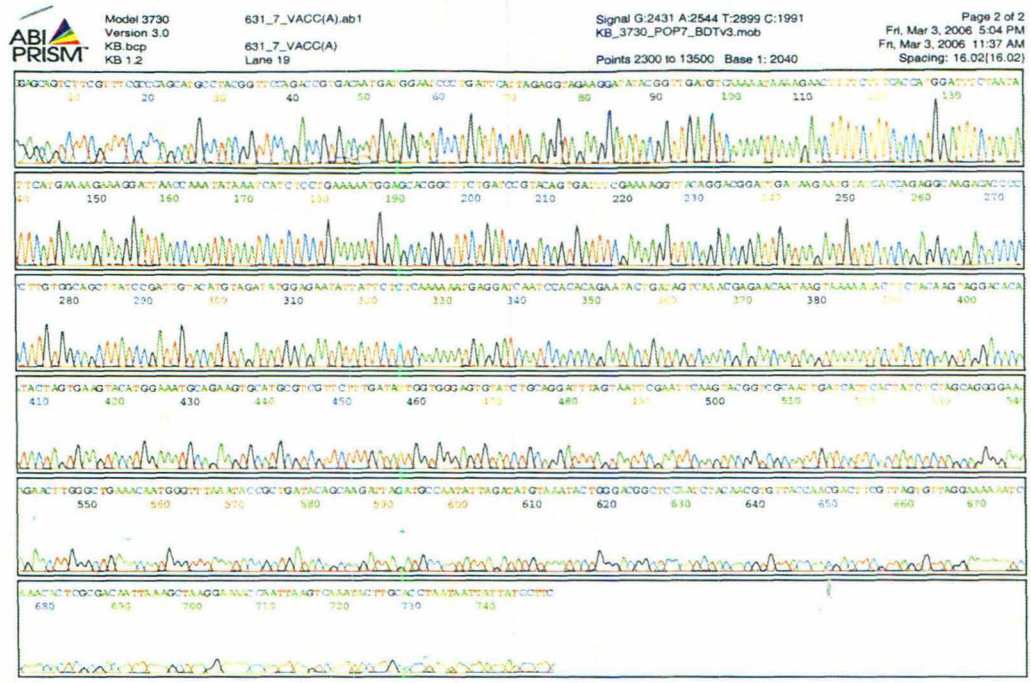
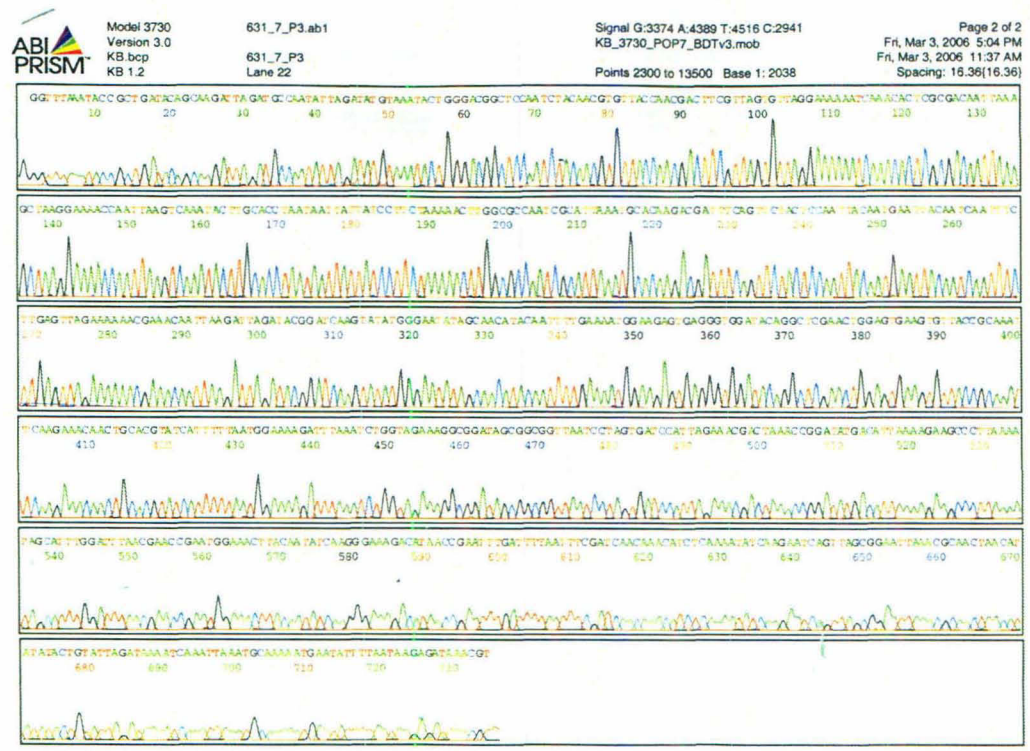
APPENDIX

Automated sequencing results of pTPA-PA63-LAMP1 clone using forward primer derived from CMV promoter region of the vector.



Automated sequencing results of pTPA-PA63-LAMP1 clone using forward primer from domain 1 of PA gene.



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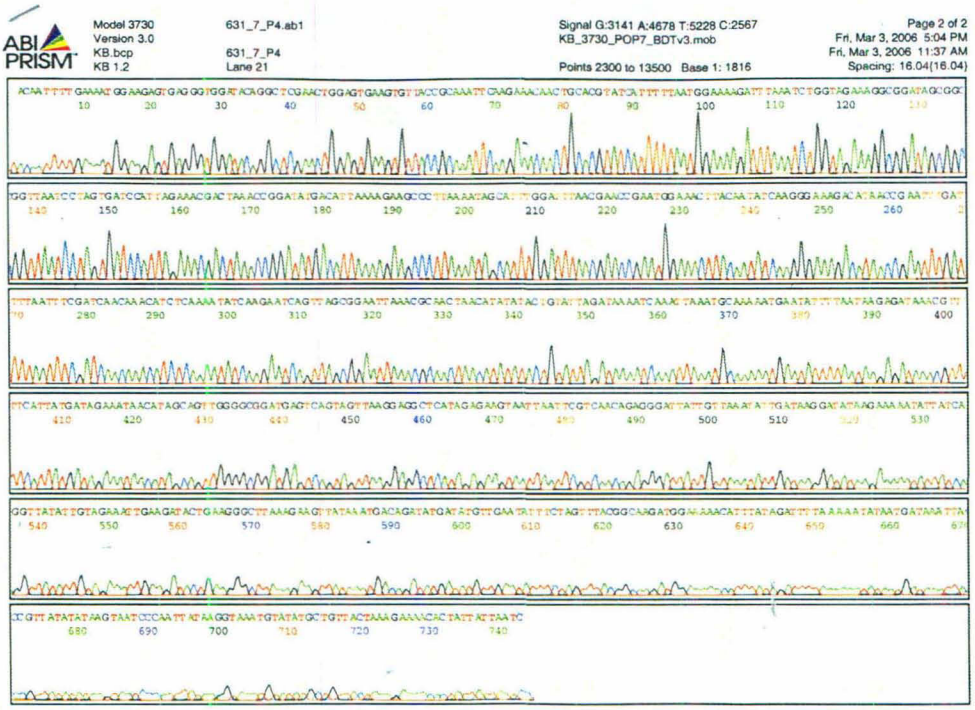

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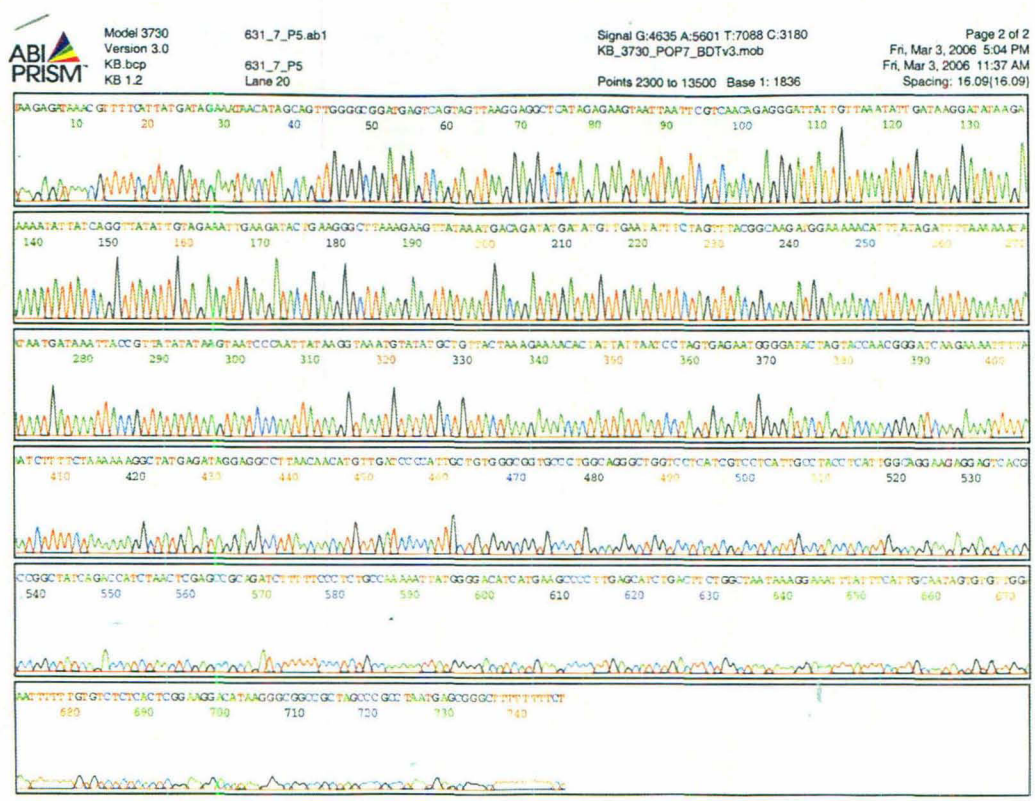

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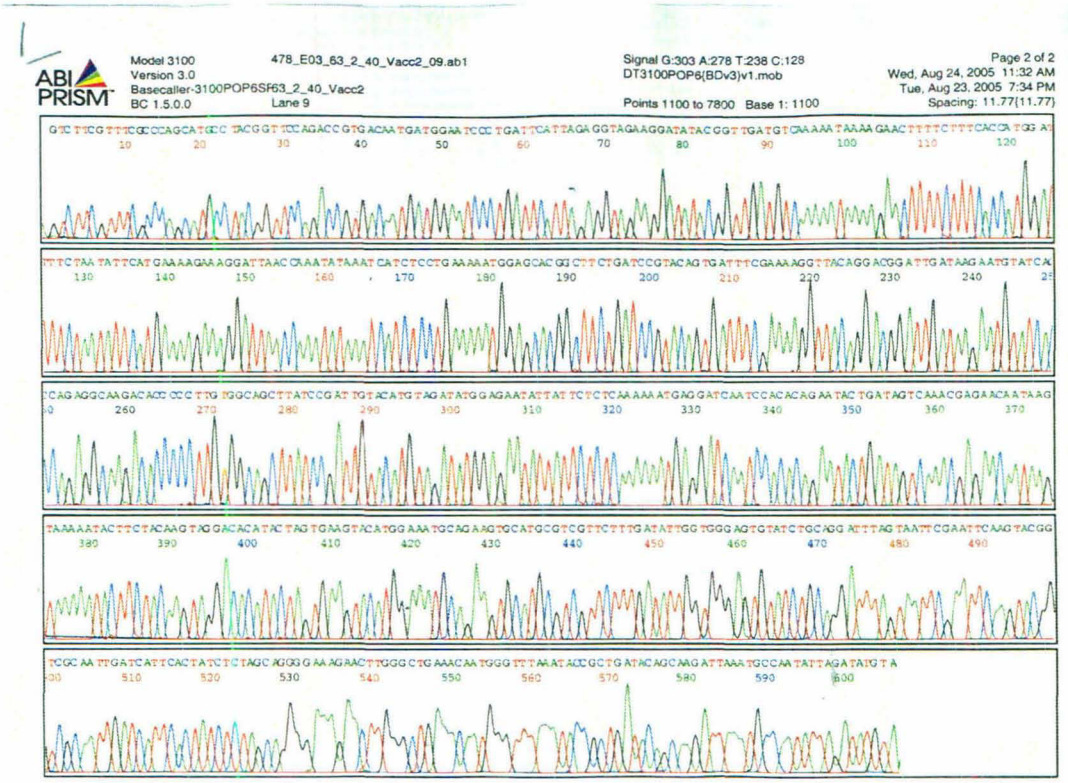

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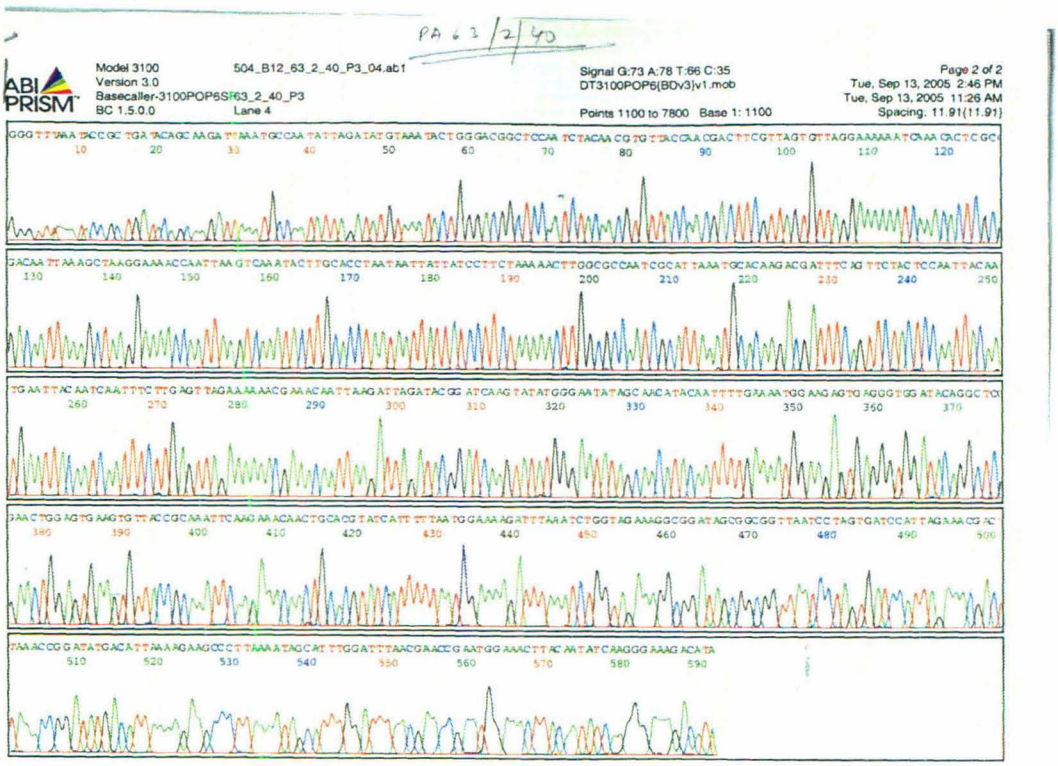

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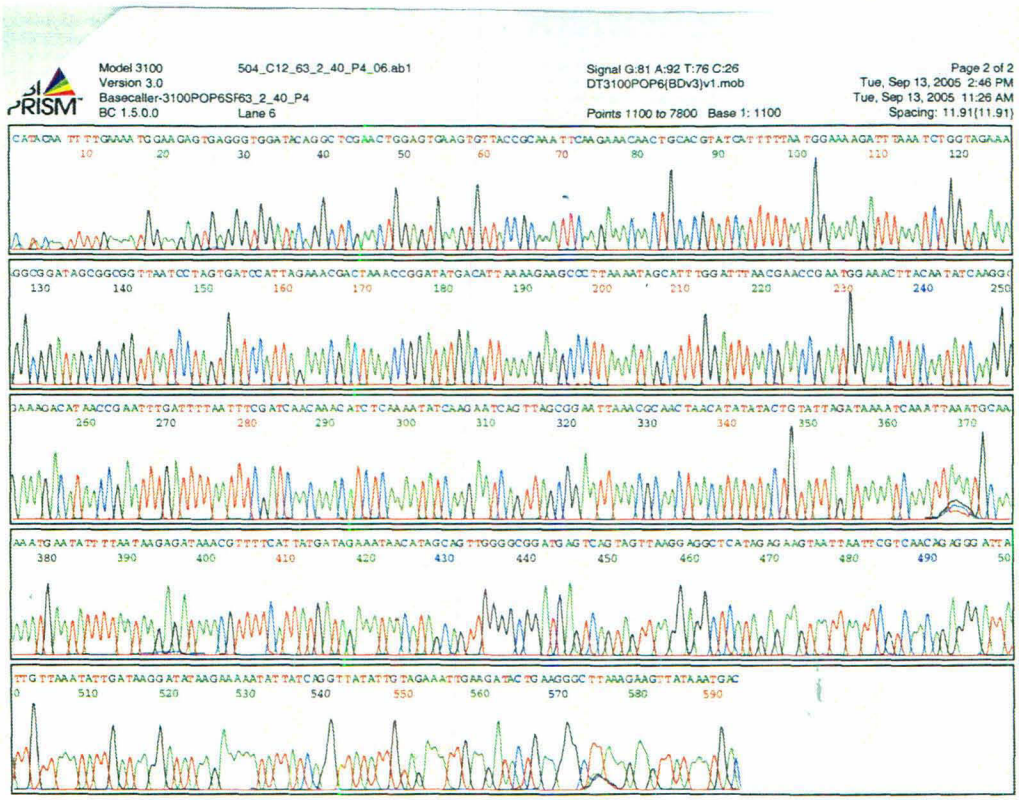

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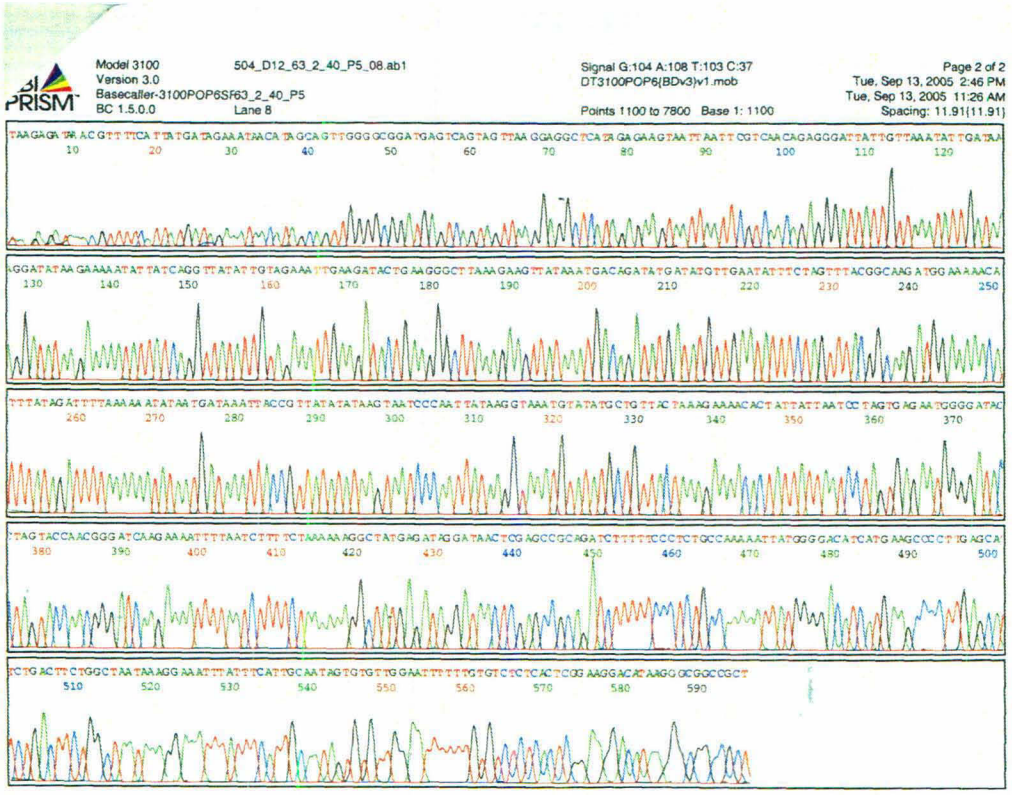
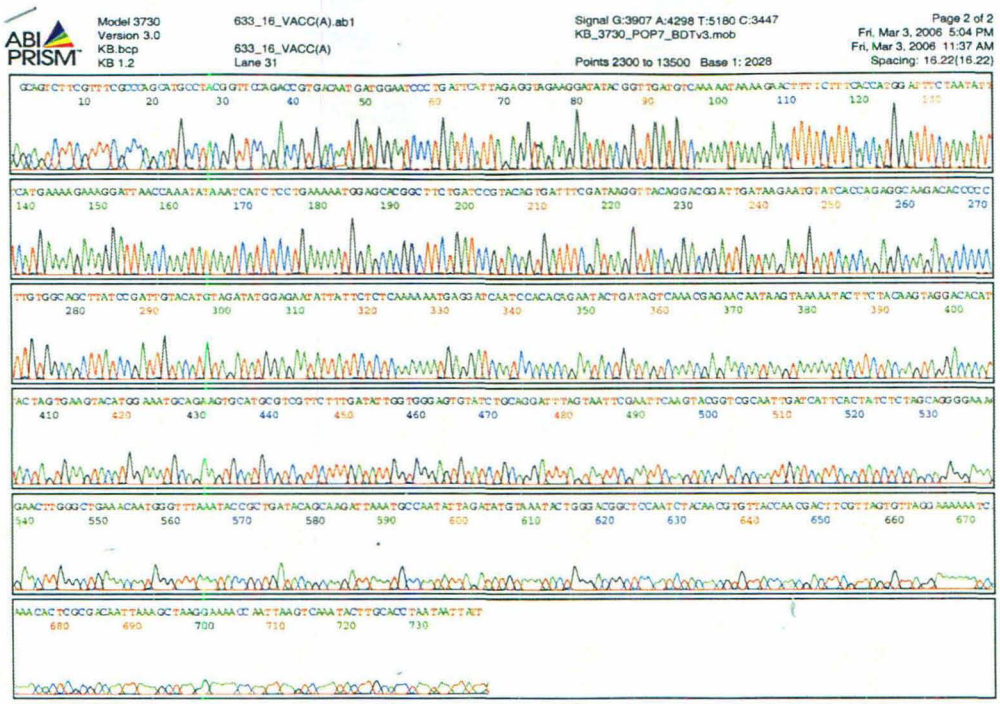


Automated sequencing results of pTPA.GPI-PA63 clone using forward primer derived from CMV promoter region of the vector.



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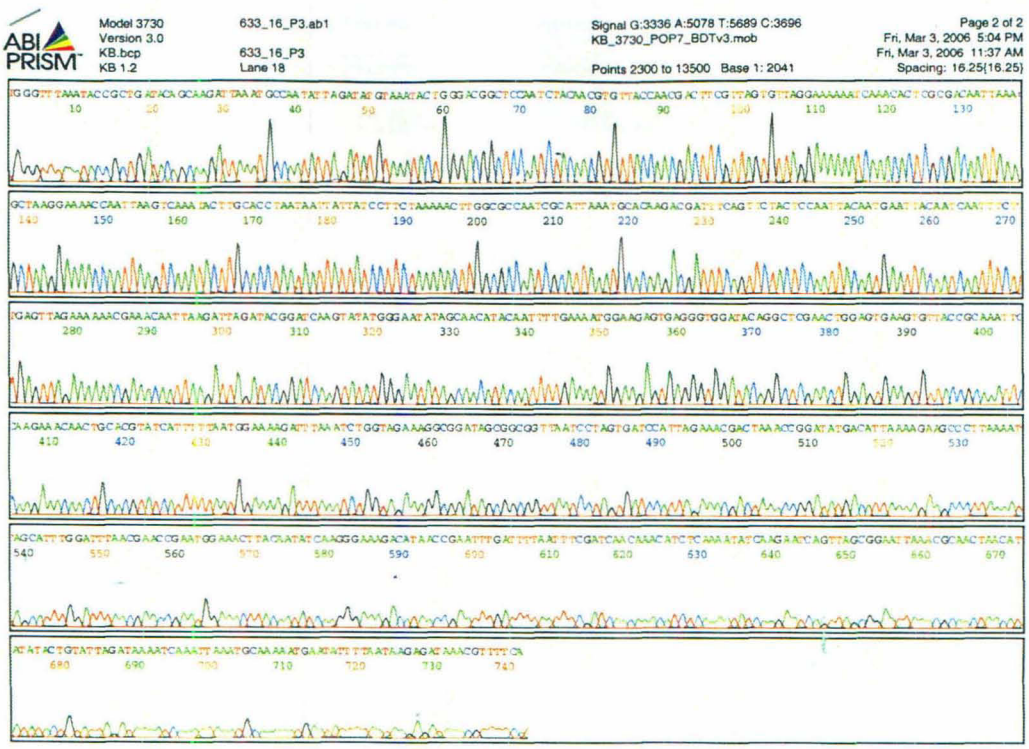

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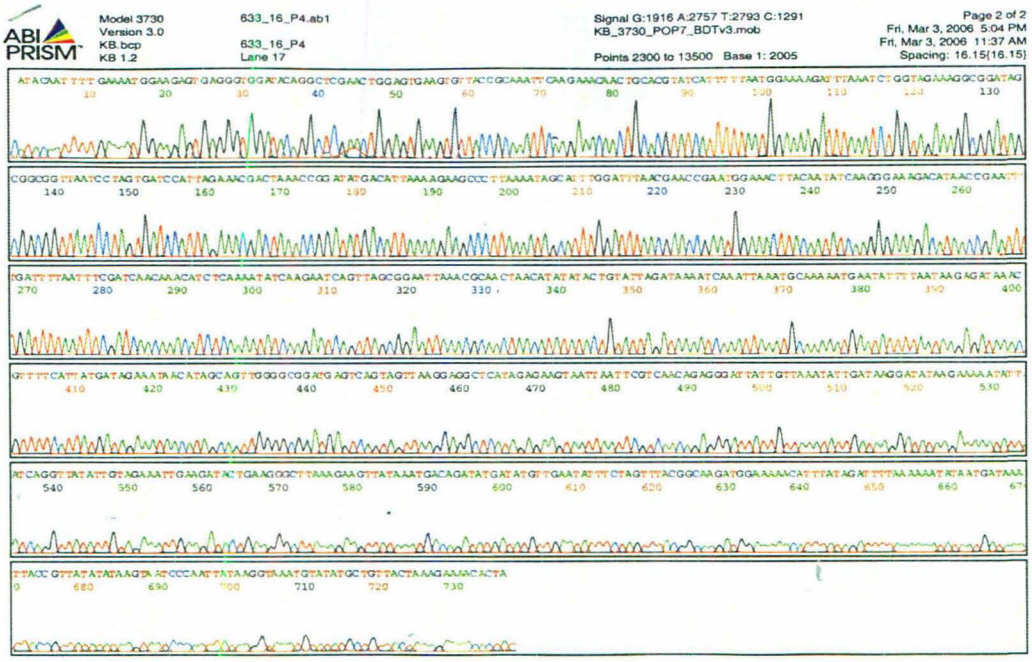

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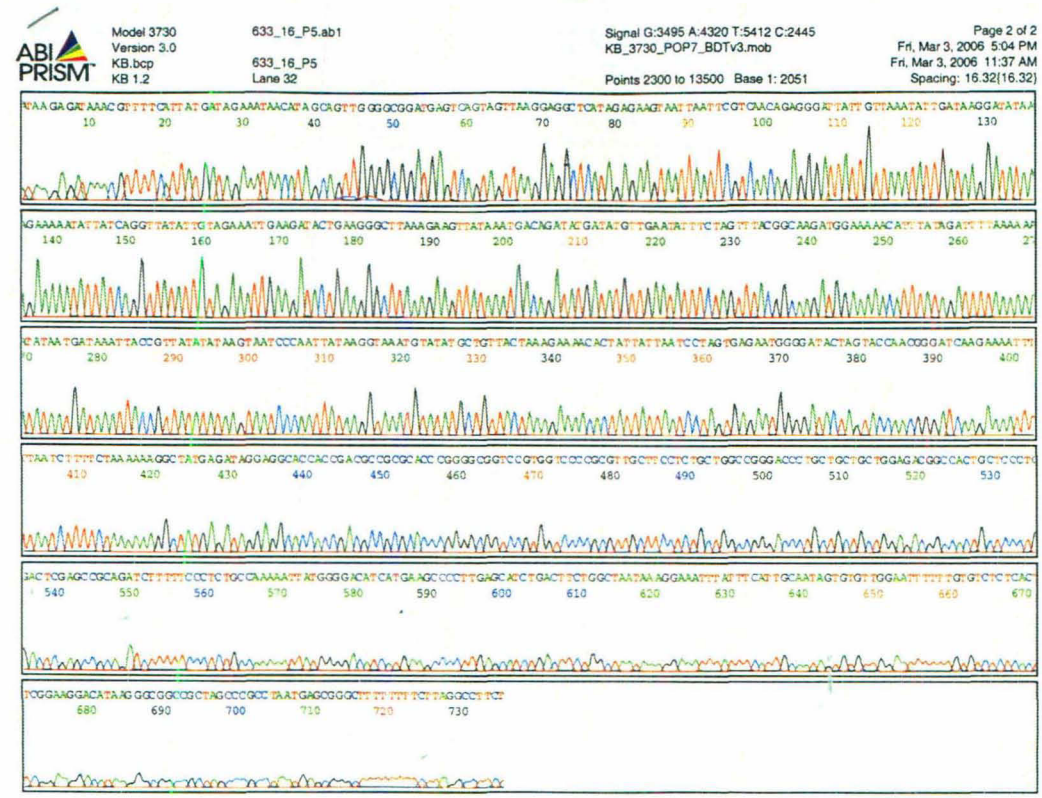
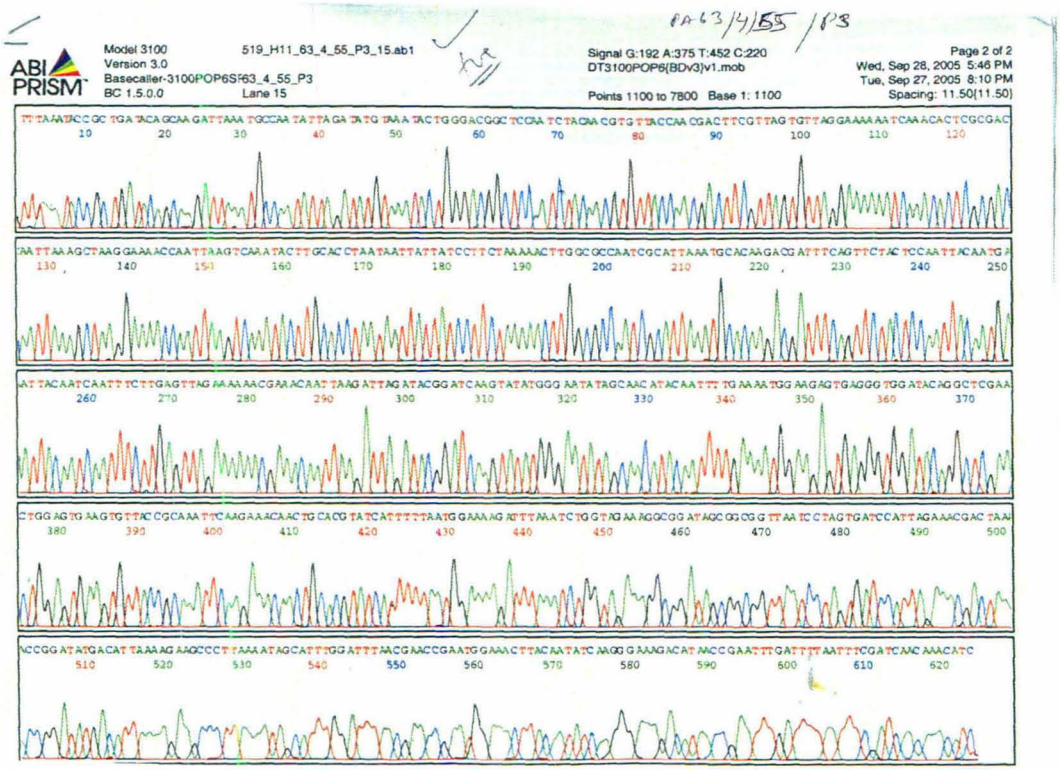


Automated sequencing results of pPA63-UQ clone using forward primer derived from CMV promoter region of the vector.



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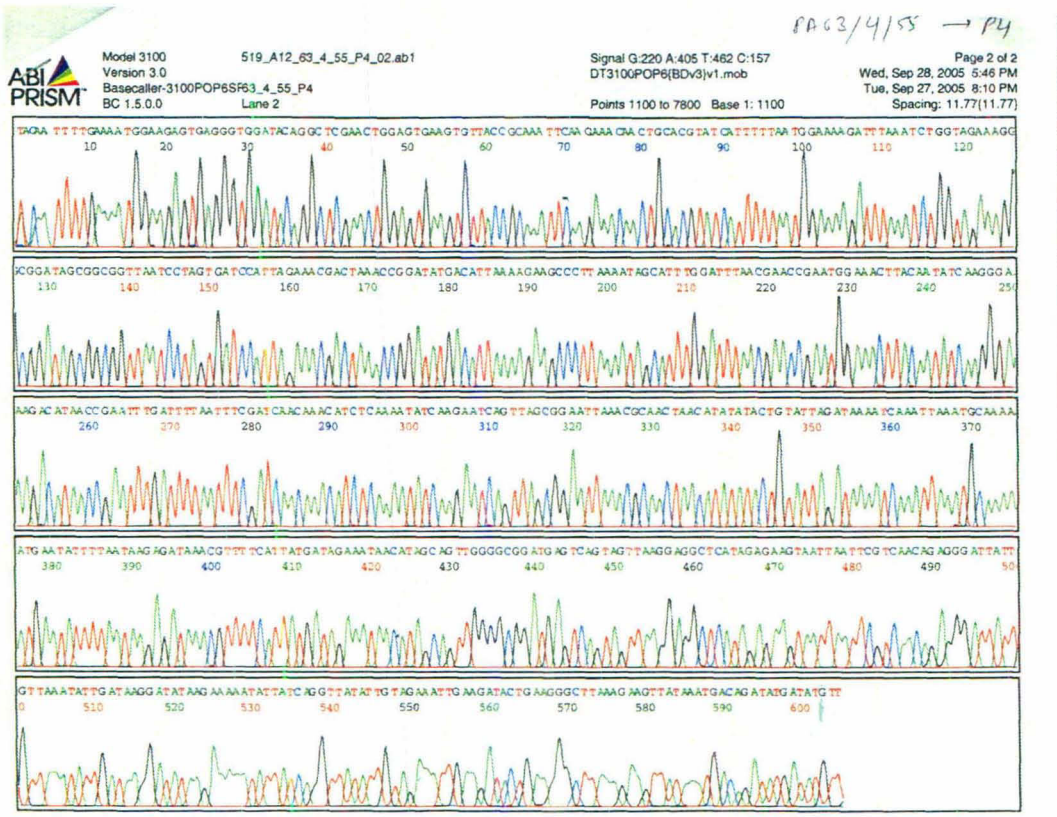

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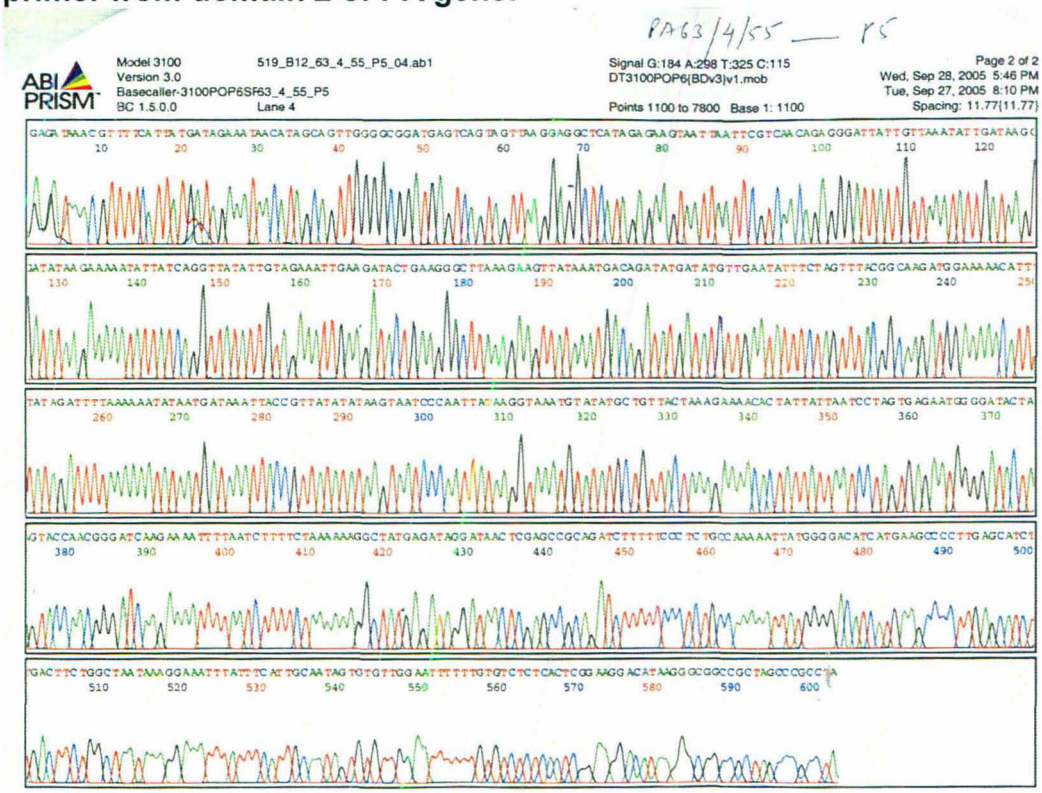
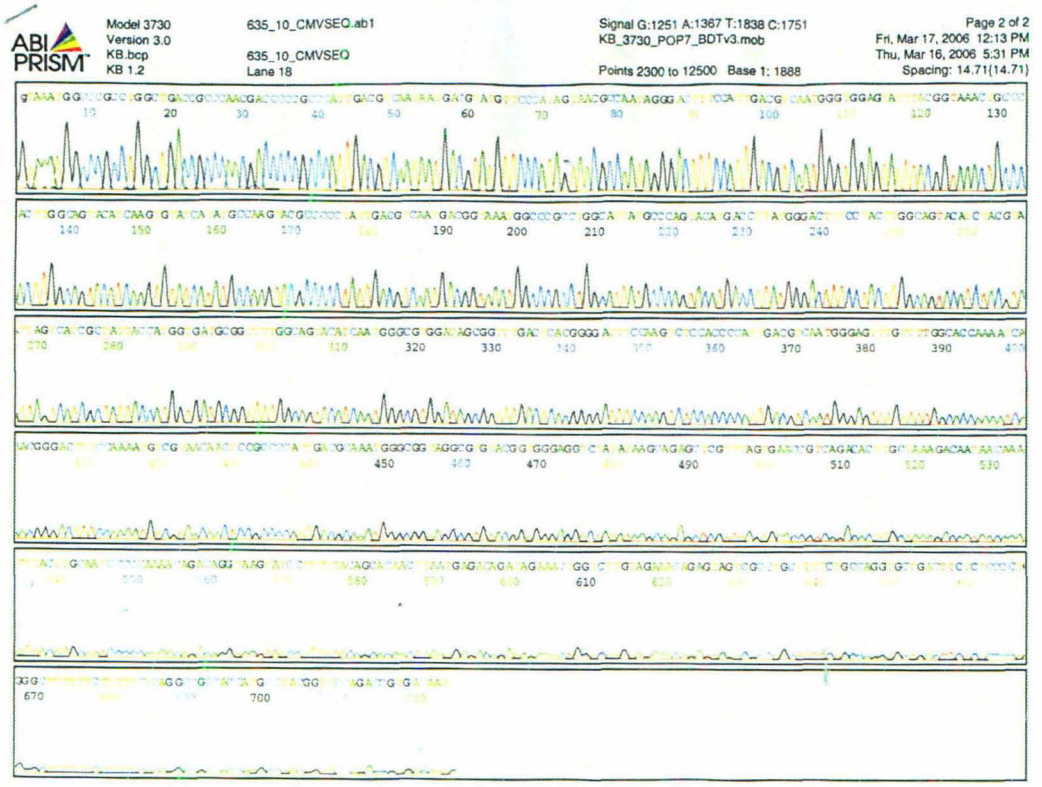
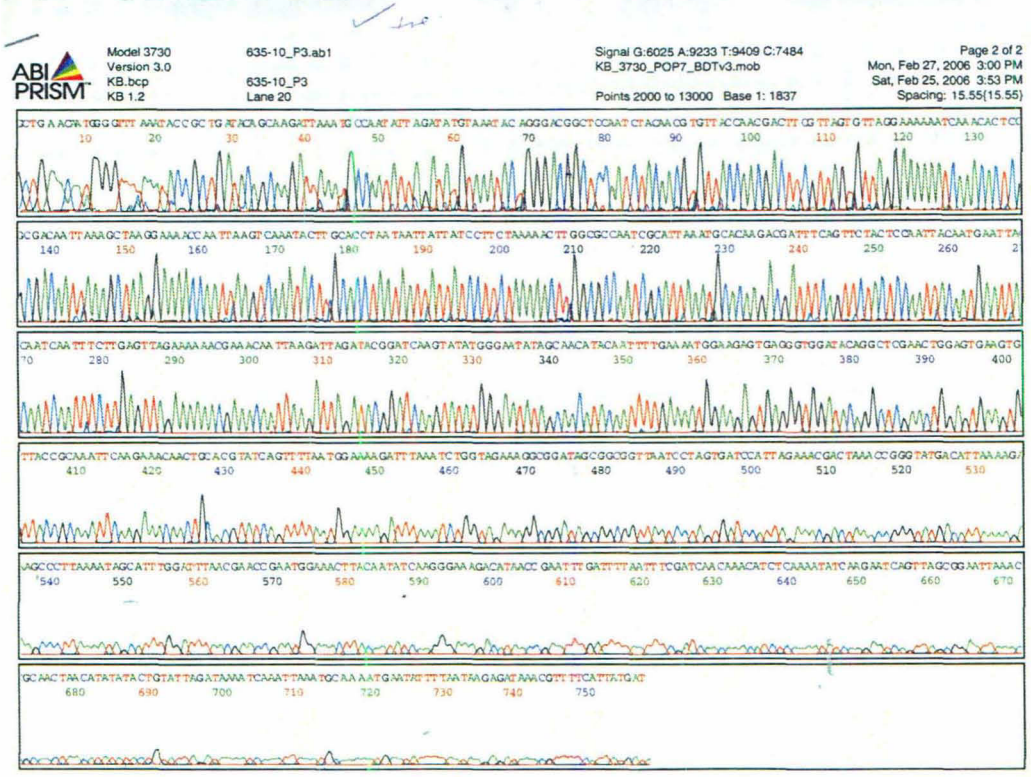


Automated sequencing results of pPA63-UQ clone using forward primer from domain 3 of PA gene.



Automated sequencing results of pPA63-Native clone using forward primer derived from CMV promoter region of the vector.



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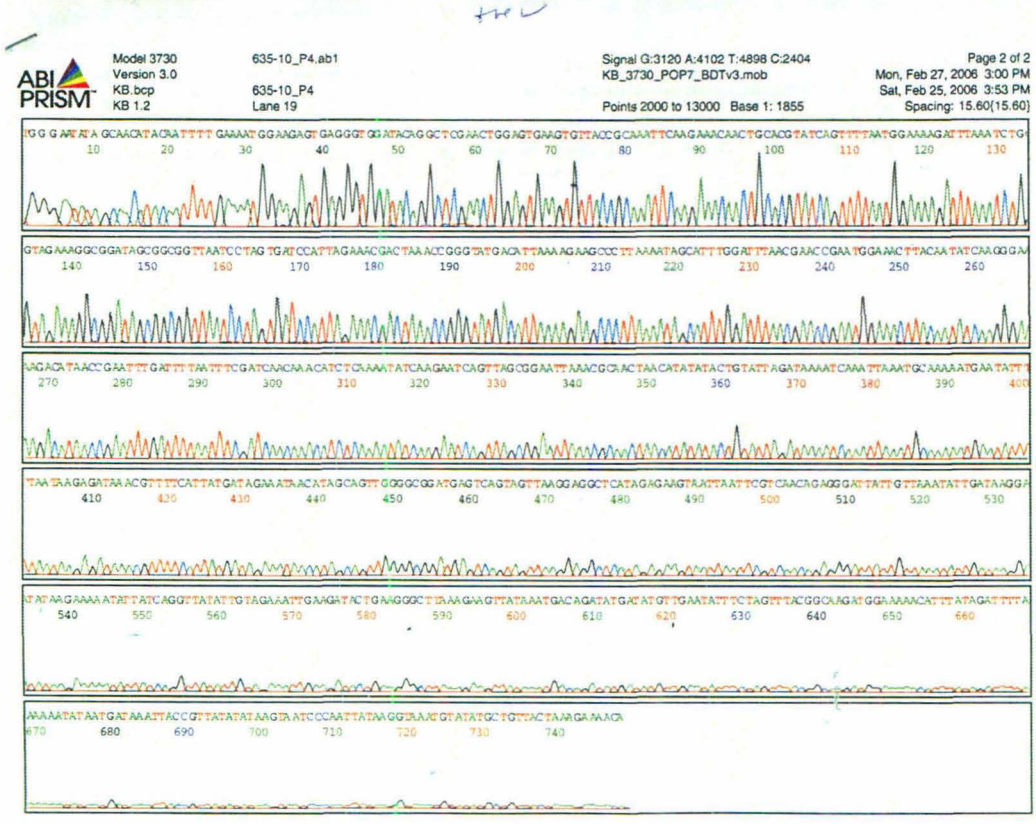

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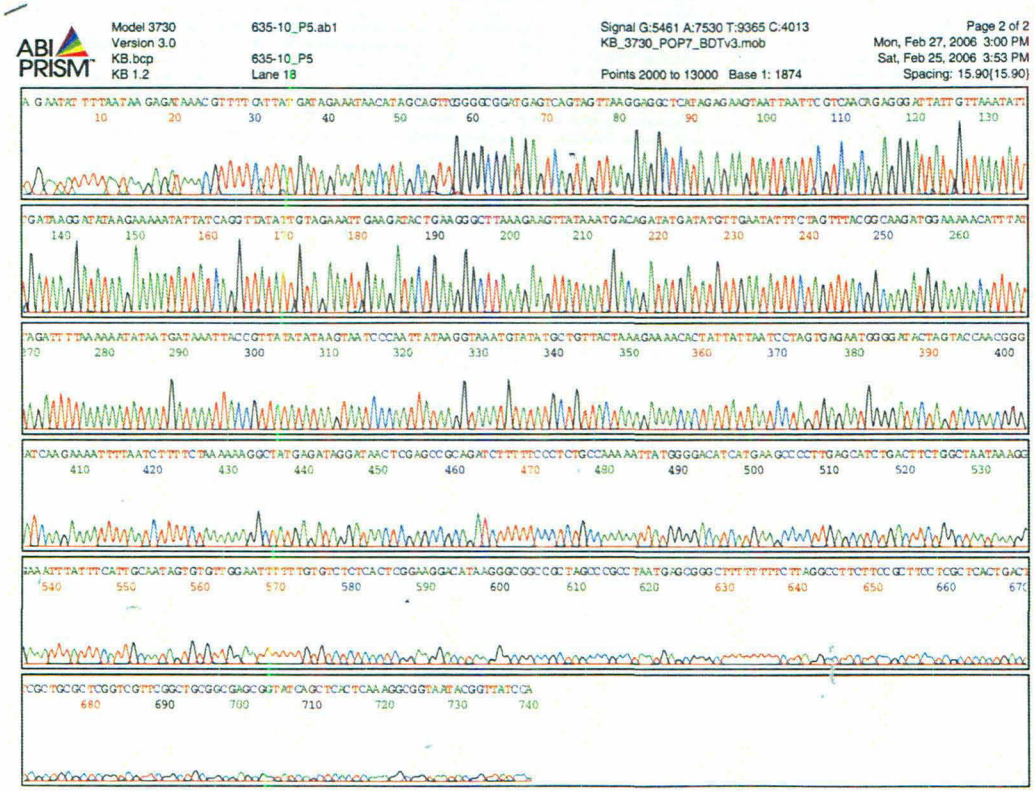

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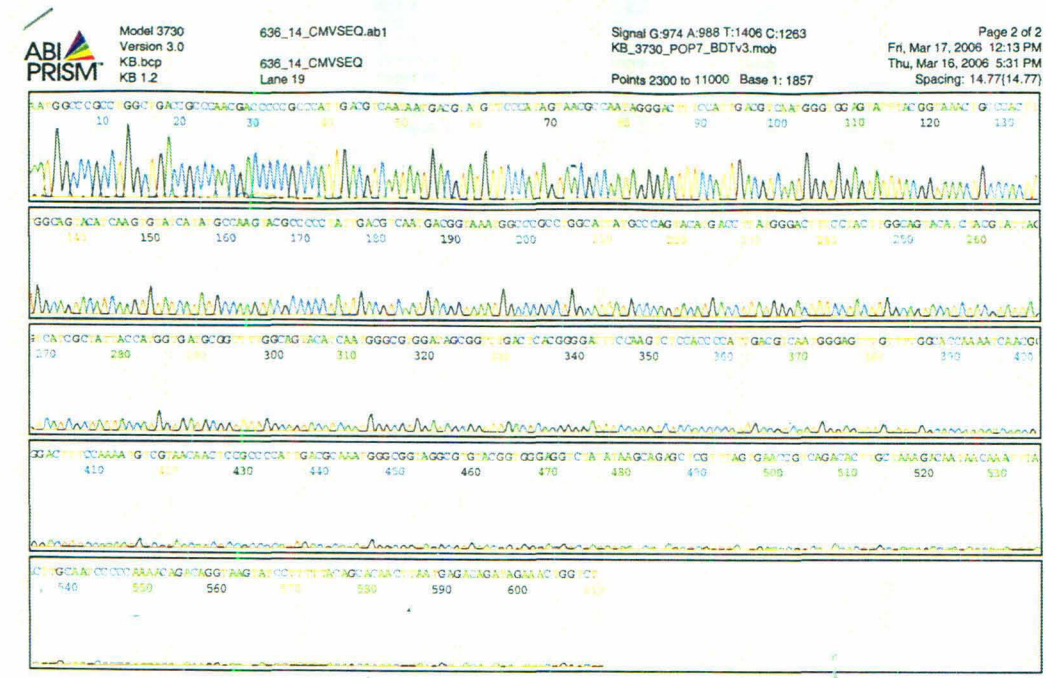
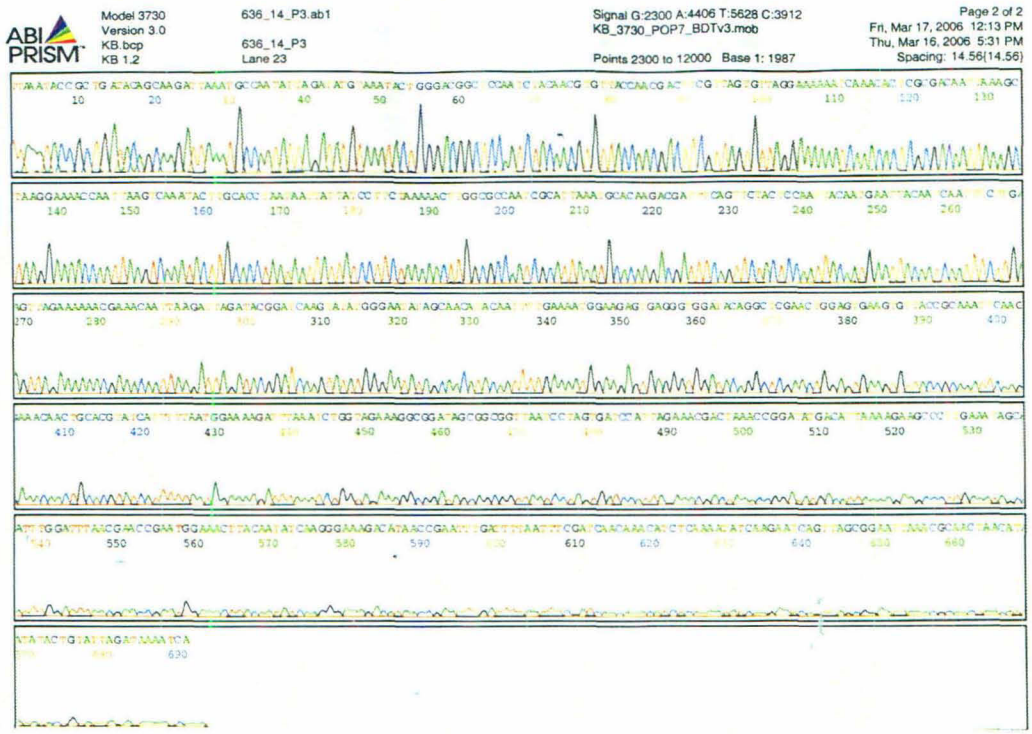
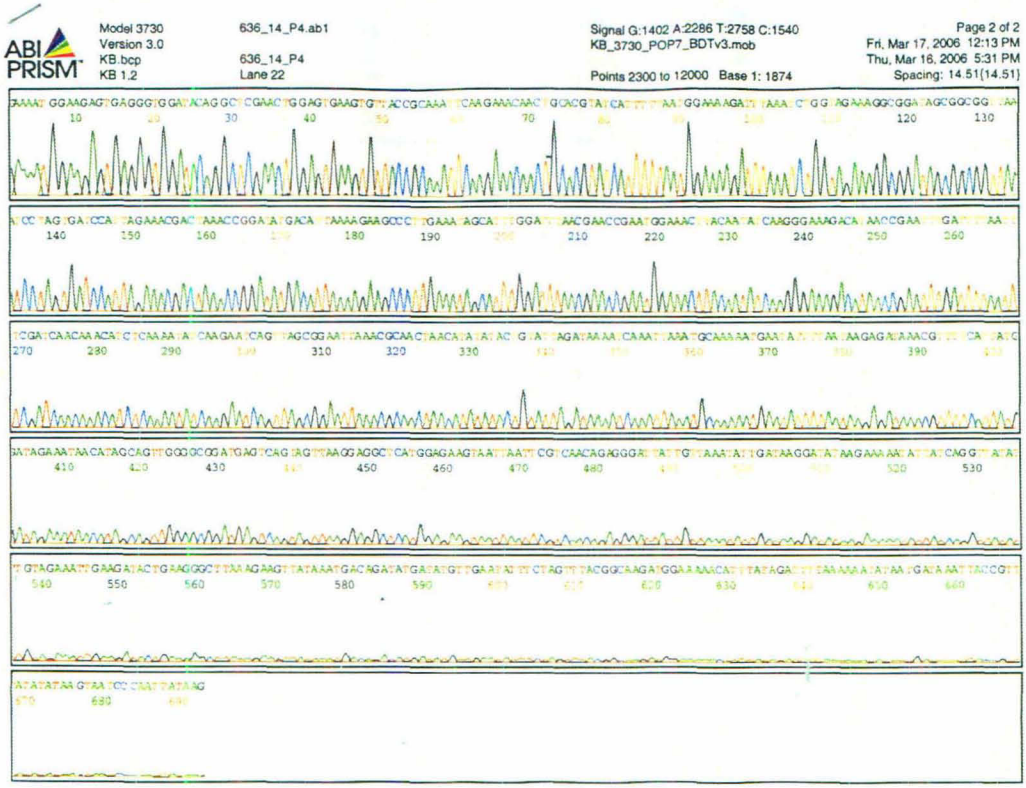


Automated sequencing results of pGPI-PA63 clone using forward primer derived from CMV promoter region of the vector.



Automated sequencing results of pGPI-PA63 clone using forward primer from domain 1 of PA gene.



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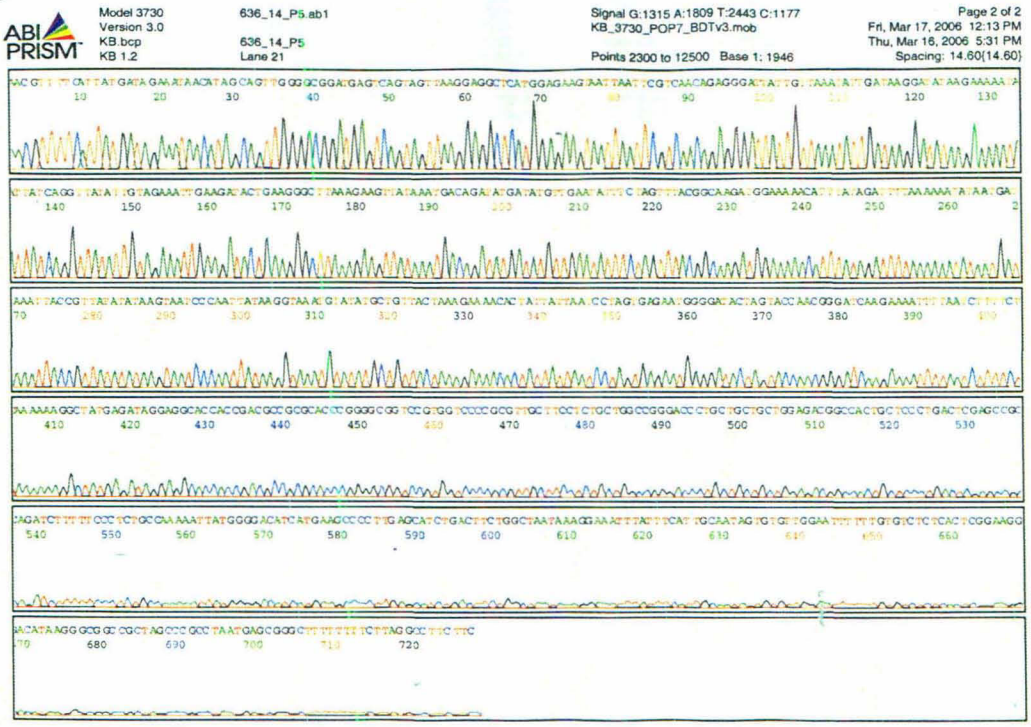
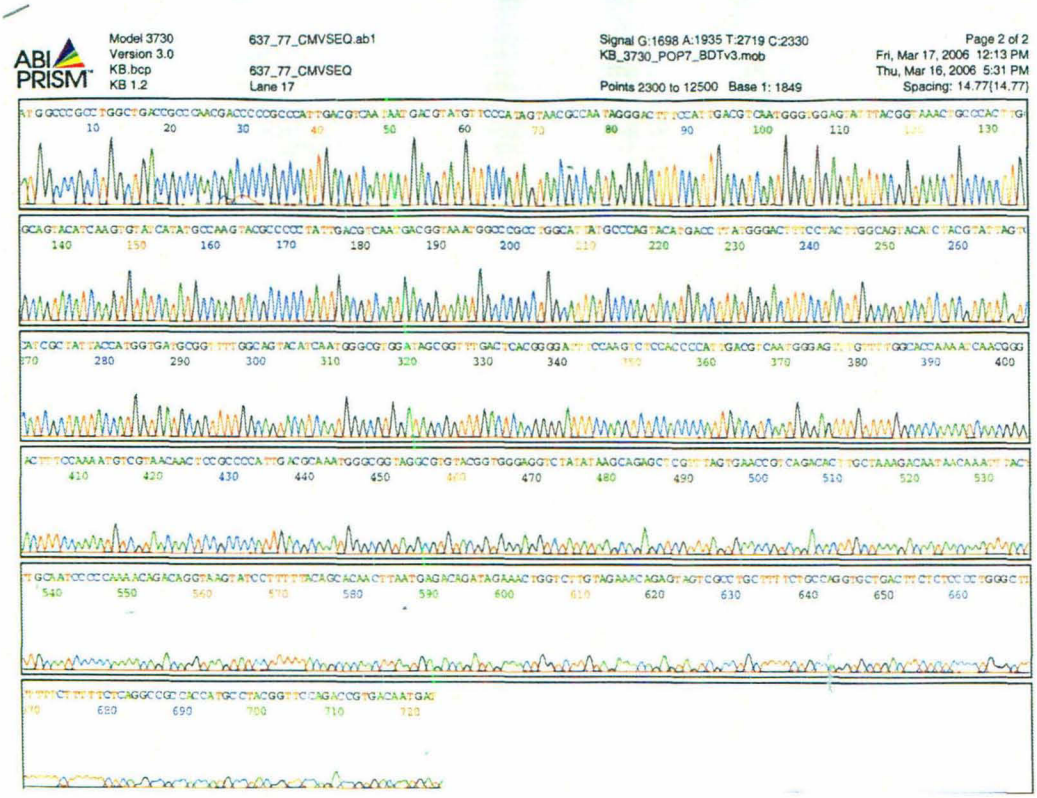
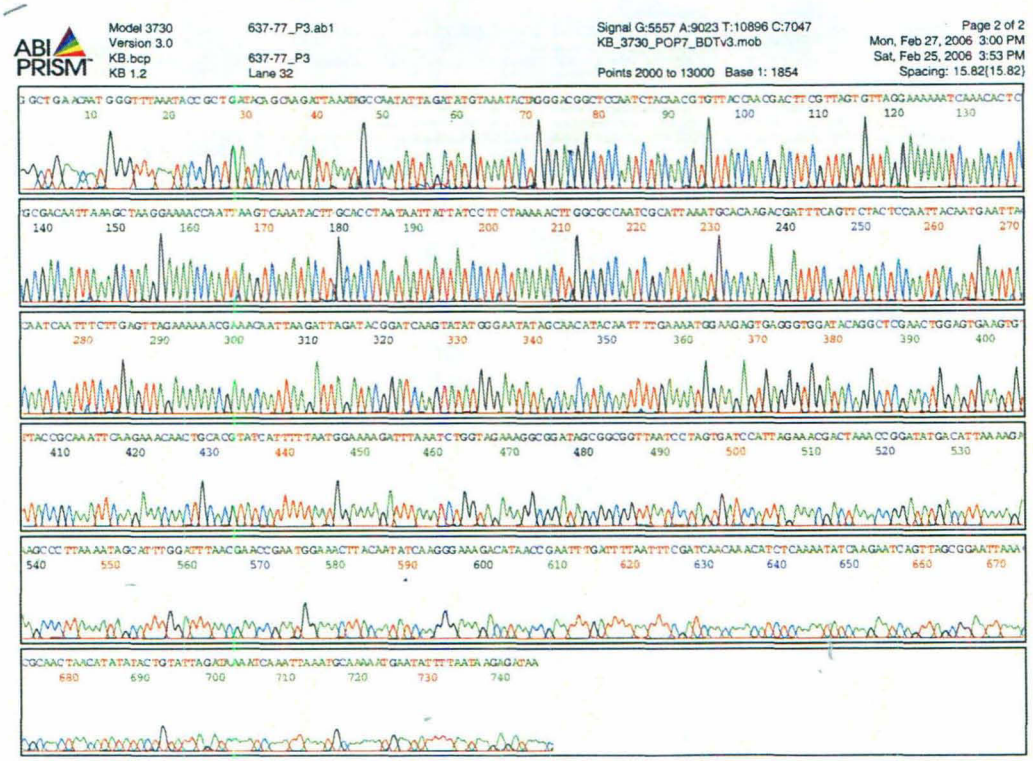


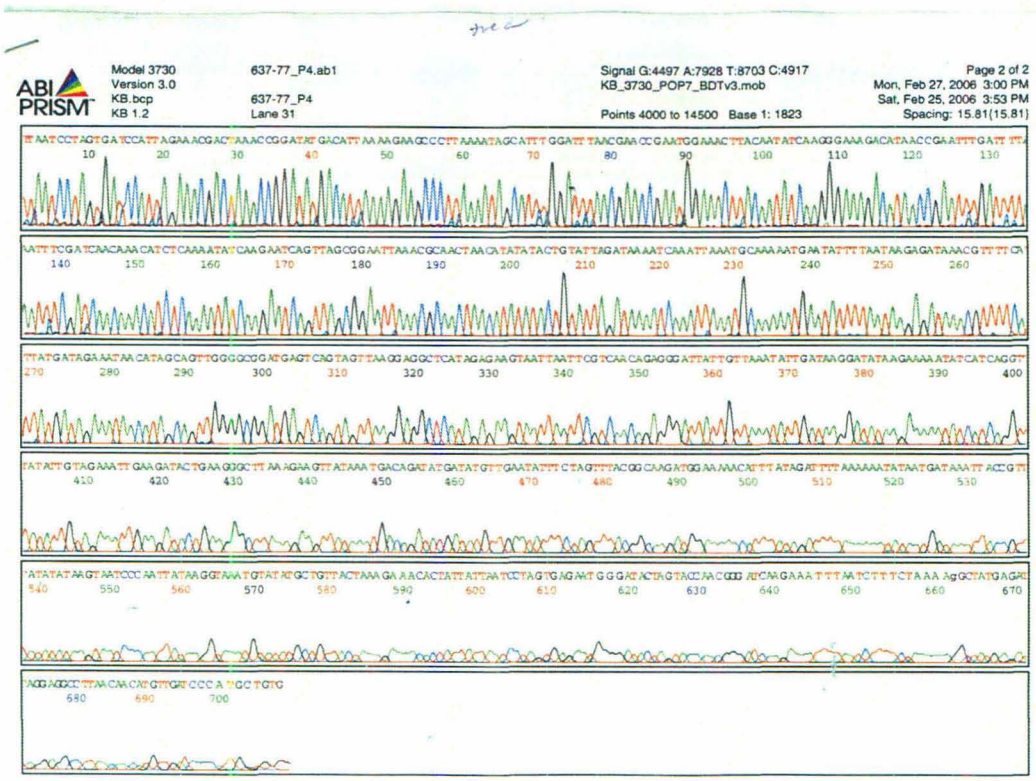
Automated sequencing results of pPA63-LAMP1 clone using forward primer from CMV promoter region.



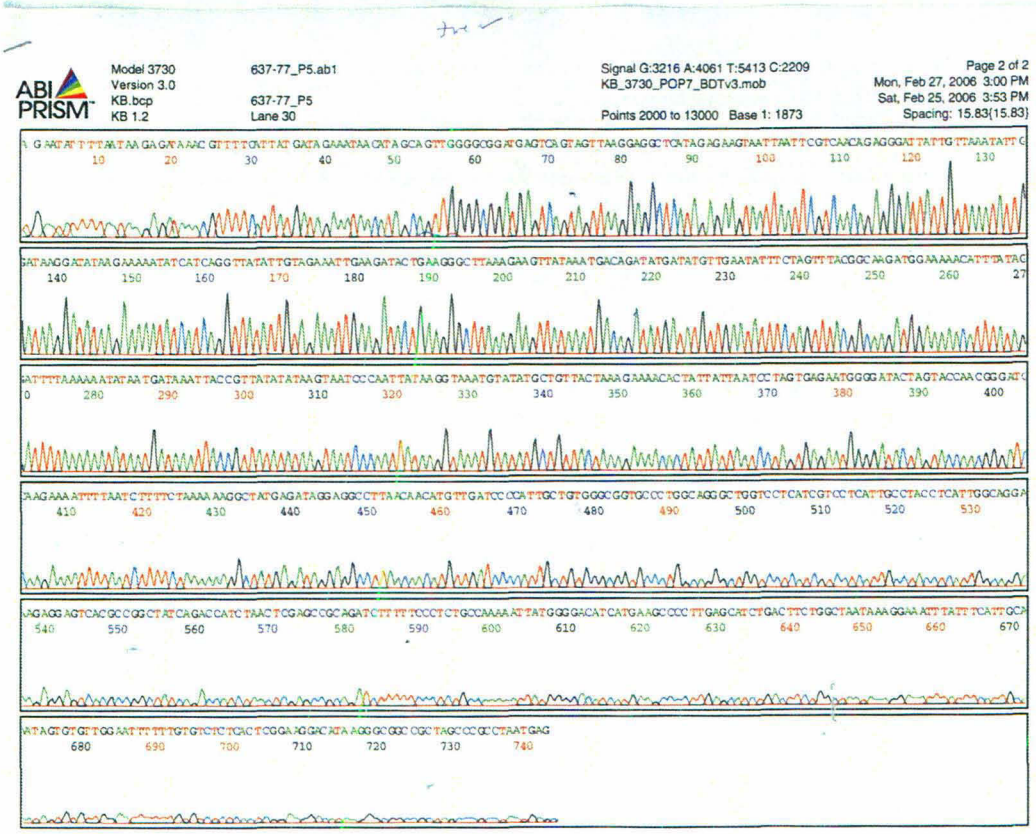
Automated sequencing results of pPA63-LAMP1 clone using forward primer from domain 1 of PA gene.



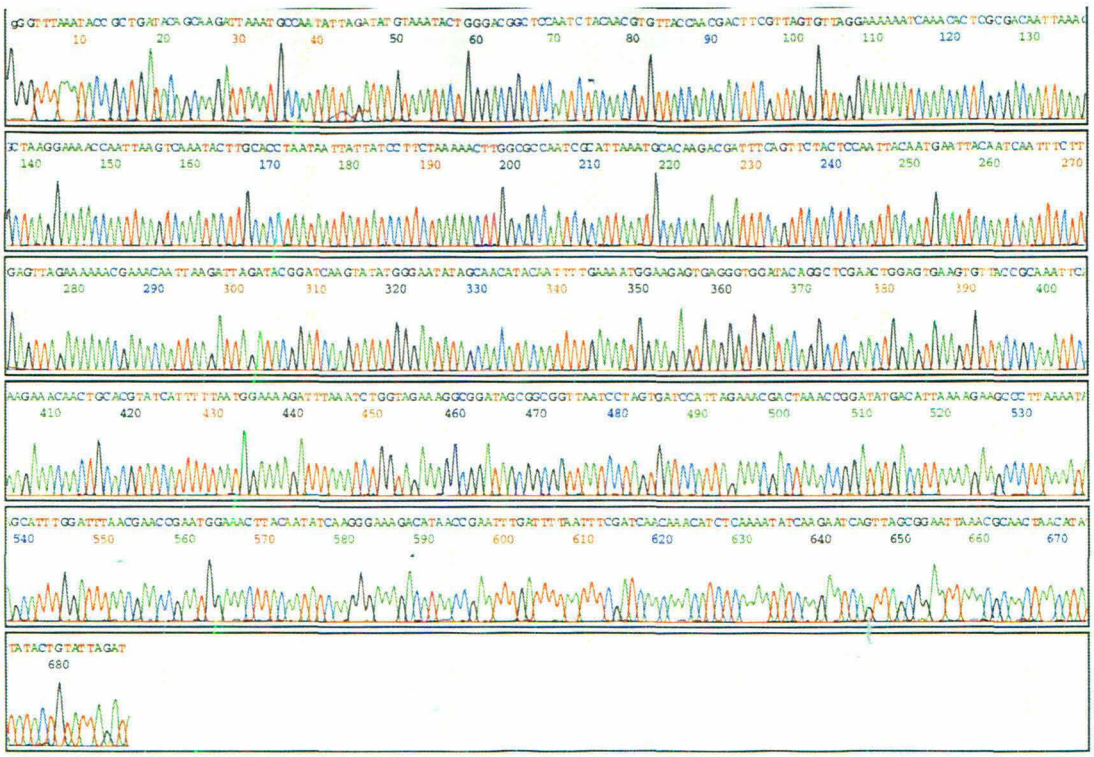
Automated sequencing results of pPA63-LAMP1 clone using forward primer from domain 2 of PA gene.



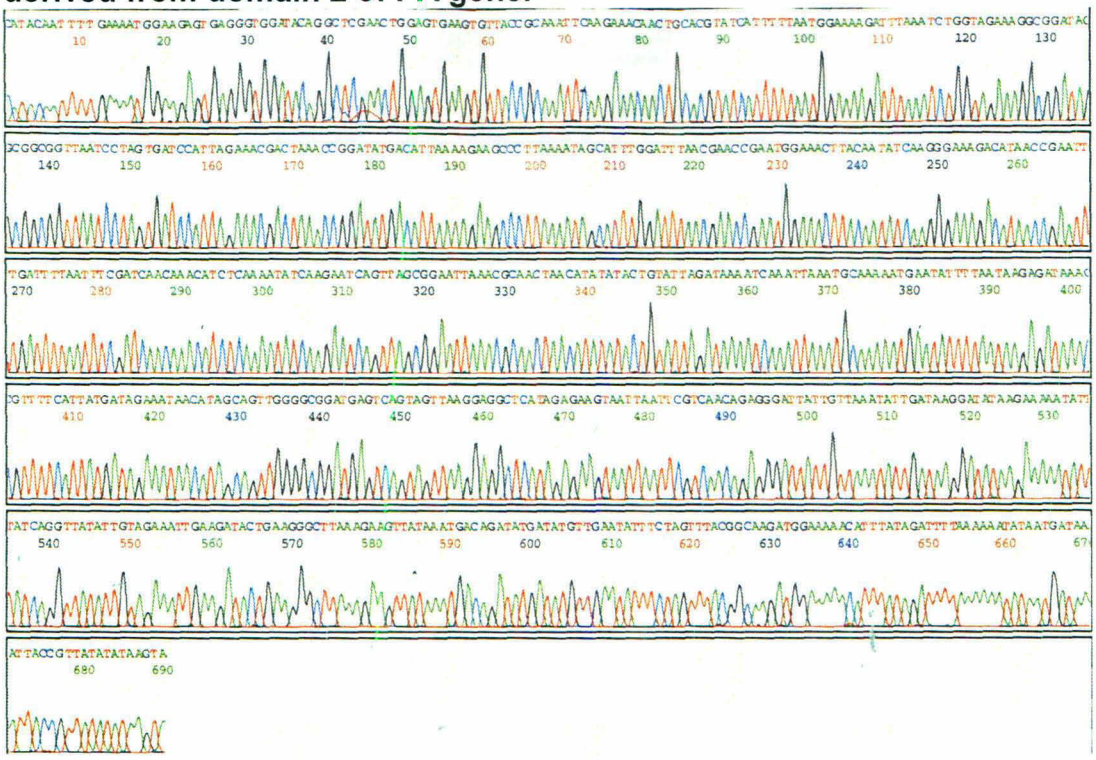
Automated sequencing results of pPA63-LAMP1 clone using forward primer from domain 3 of PA gene.



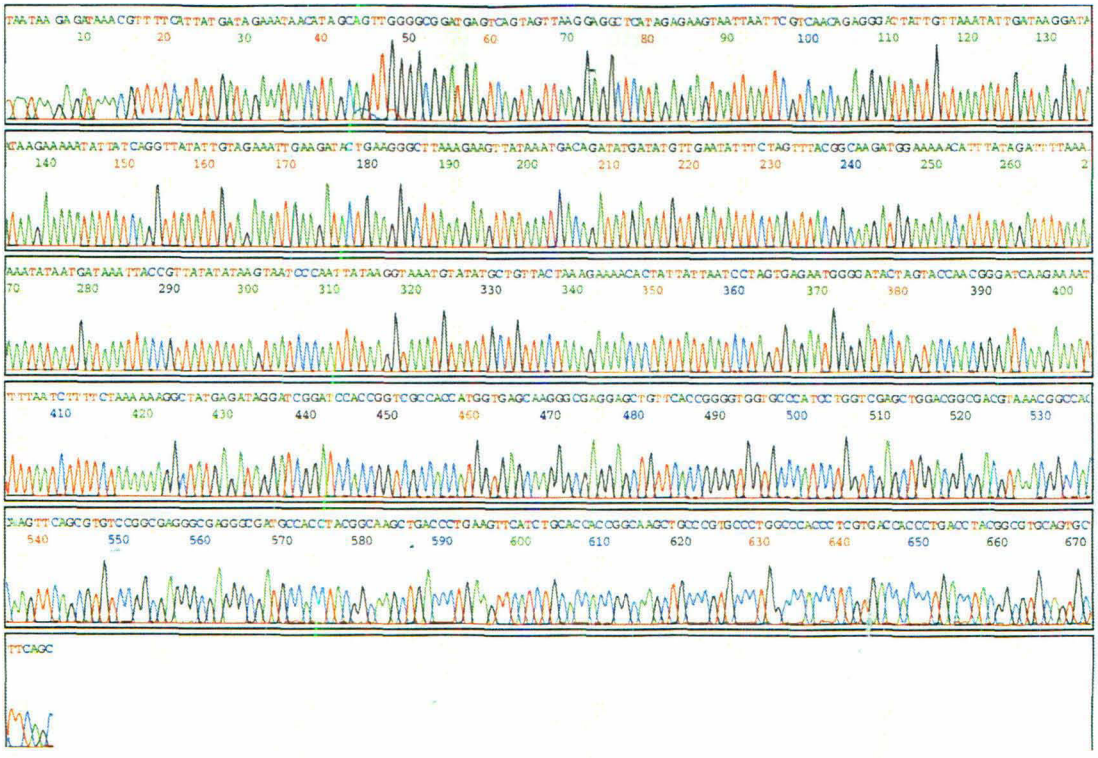
Automated sequencing results of prPA63 clone using forward primer derived from T7 promoter region of pQE30 vector.



Automated sequencing results of prPA63 clone using forward primer derived from domain 2 of PA gene.



Automated sequencing results of prPA63 clone using forward primer derived from domain 3 of PA gene.



PUBLICATIONS

the main immune evasion strategies of *Bacillus anthracis*. Cellular intoxication begins when PA (protective antigen, 83kDa), the receptor binding moiety binds to cell surface exposed anthrax toxin receptor on mammalian cells [5, 6]. Further upon furin cleavage, the protease-cleaved fragment of protective antigen (PA63) forms a pre-pore with a β-barrel structure, resulting from association of individual monomer loops [7-9]. This pre-pore then binds to LF (Lethal factor) and EF (Edema factor) and the resulting complex gets subsequently internalized by endocytosis. Inside the endosome, pre-pore to pore conversion is triggered by the low pH in this compartment which ultimately mediates the translocation of LF and EF into the cytosol [10-12]. Once inside cytosol, LF, a zinc metalloprotease, proteolytically cleaves short N-terminal fragments from mitogen or extracellular signal-regulated protein kinase kinase1 (MEK1), MEK2 and MEK3, the upstream activators of ERK1, ERK2 and p38, respectively. As a result, antigen receptor signaling gets disrupted which causes macrophage function inhibition [13-15] and suppression of T-lymphocyte activation [16]. On the other hand, EF, an adenylate cyclase, upon calmodulin binding undergoes structural rearrangements that leads to its activation and induces substantial increase in conversion of intracellular ATP to cAMP [16]. Subsequently, water homeostasis and cellular signaling of the host are disrupted, leading to edema during cutaneous anthrax infection [17, 18]. Additionally, EF inhibits the ability of neutrophils to phagocytose the bacilli and produce oxidative burst [19, 20]. It also co-operates with LF to impair cytokine secretion during the infection of dendritic cells which ultimately leads to suppression of innate immunity [21].

Therefore, the current scenario necessitates retrospection and design and development of an effective alternative approach for prophylaxis. In this context DNA vaccines seem to hold an enormous potential. Immunization with DNA vaccine encoding the antigen (Ag) of interest has been used to induce both cellular and humoral immunity. Reports of the first human clinical trials have shown that DNA vaccines are well tolerated and the results have been encouraging [22-26]. Although the immunological correlates of protection against anthrax have yet to be defined, several studies in non-human primates support the correlation between vaccine-induced neutralizing antibodies against PA and protection against subsequent challenge with pathogenic *B.anthracis* strain [27-31]. In addition, protection mediated by PA has been shown to be T-cell dependent [32, 33].

In this regard, DNA vaccination strategies have either relied on the usage of various adjuvants like, cation-lipids [34, 35], CpG oligonucleotides [36]; or else on DNA delivery by, gene-gun [37], liposomes [38], poly-coglycoside particles [39], with an aim to enhance the cellular as well as humoral immune responses. Systemic vaccination strategies based on prime boost regimen that imply a DNA prime followed by a booster with recombinant PA have been tested [40, 41]. Additionally, DNA vaccine encoding a fragment of LF has been shown to provide protection against lethal toxin challenge [42]. Although these vaccine regimes were tested for their ability to induce measurable antibody responses, the generation of effective cellular adaptive immunity arising as a result of DNA vaccination was not investigated.

Given these considerations we report here the application of antigen trafficking to various compartments of the cell with an ultimate goal of improving the humoral as well as cellular immune responses. There is an increasing body of evidence to suggest that both $CD8^+$ and $CD4^+$ T cells are critical for the generation of an effective immune response against an intracellular pathogen. Although both recognize non-native forms of the antigen in association with major histocompatibility complex (MHC) molecules, the presentation of the antigen to these two lymphocytic populations occurs through distinct pathways [43]. For processing through the MHC II pathway the antigen needs to be targeted to the endosomal or lysosomal compartment [44, 45]. The traditional pathway for antigen targeting to lysosomal compartment involves phagocytosis or endocytosis of the exogenous antigen or specific targeting to lysosomal pathway by attachment of lysosome targeting signals. Based on these observations we reasoned that a molecular approach that enroutes the antigen to the MHC II pathway might facilitate endogenous presentation to $CD4^+$ T cells. At steady state, immunoreactive LAMP1 (lysosome associated membrane protein) is highly enriched in the late endosomes and lysosomes [46, 47]. LAMP and MHC II are closely linked in their co-localization in MIIC (for MHC II containing compartments) [48]. Also, TPA leader sequence (Tissue plasminogen activator) has been shown to facilitate the secretion of the encoded antigen [49]. Therefore, its inclusion in DNA construct may help facilitate secretion of vaccine-encoded antigen that could well be taken up as an exogenous antigen by

European Journal of Immunology

Potentiation of humoral and cellular responses following delivery of 63kDa fragment of anthrax Protective antigen via targeted DNA vaccination to distinct subcellular locations.

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Based on the hypothesis that immune outcome can be influenced by the form of antigen administered and its ability to access various antigen-processing pathways, we mediated the targeting of 63kDa fragment of protective antigen (PA) of *Bacillus anthracis* to various subcellular locations by DNA chimeras bearing a set of signal sequences. These targeting signals namely, LAMP1 (Lysosome Associated Membrane Protein 1), TPA (Tissue Plasminogen activator) and Ubiquitin encoded various forms of PA viz. lysosomal, secreted, and cytosolic, respectively. Examination of IgG subclass distribution arising as a result of DNA vaccination indicated a higher IgG1:IgG2a ratio whenever the groups were immunized with chimeras bearing TPA, LAMP1 signals alone or when combined together. Importantly, high end-point titers of IgG antibodies were maintained until 22 weeks. It was paralleled by high avidity toxin neutralizing antibodies (TNA) and effective cellular adaptive immunity in the systemic compartment. Anti-PA and TNA titers of $\approx 10^5$ and $\approx 10^3$, respectively, provided protection to ≈90% of vaccinated animals in the group pTPA-PA63-LAMP1. A significant correlation was found between survival percentage and post-challenge anti-PA titers and TNA titers. Overall, immune kinetics pointed that differential processing through various compartments gave rise to qualitative differences in the immune response generated by various chimeras.

Keywords
- DNA vaccination
- Anthrax
- Protease-cleaved
- Fragment
- Protective antigen

INTRODUCTION

Anthrax bioterror attacks of 2001 reinstated interest in *Bacillus anthracis*, the etiological agent of anthrax. Since then concerted efforts have been made in the direction of effective vaccine development, antitoxin-treatment strategies, and development of advanced diagnostics, for prevention, cure and detection respectively [1, 2]. However, no significant breakthrough has been achieved as yet. Pre-exposure vaccination and post-exposure antibiotic therapy serve to be the only defense [2]. Besides that the currently available anthrax vaccines suffer from the drawback, in being relatively reactogenic and having a long dosage schedule which requires frequent administration of boosters [3, 4]. Therefore, there is an urgent need to develop a safe, affordable and efficacious vaccine against anthrax.

Serious attempts have been made in the past to unveil the mechanism of host immune paralysis adopted by *Bacillus anthracis*. Gathered evidences point towards toxin-mediated immuno-modulation of host immune response, necrosis or apoptosis of various host cell types and, capsule-mediated inhibition of phagocytosis being

phagocytosis or endocytosis by APC and directly targeted to endosomal pathway. In addition to efficient antigen targeting and presentation of antigen via the MHC II pathway, effective $CD8^+$ T cell responses are dependent upon efficient processing through MHC I pathway alongwith the help provided by $CD4^+$ T cells [50]. For that we took advantage of the fact that carboxy terminus of UQ (Ubiquitin) is a substrate for a site-specific protease [51]. Rationale was, ubiquitination should target the protein for rapid cytoplasmic degradation by the proteasome and increase the availability of the antigenic peptides for presentation through the MHC I pathway [52]. Subsequently, immune recognition should be reflected in the magnitude of Ag-specific $CD8^+$ T cell-mediated immune responses generated.

We therefore utilized a set of gene targeting sequences that are (Table1) capable of targeting PA63 to various sub-cellular locations. The results demonstrated that the magnitude of immune response was dependent on the cellular location of the expressed antigen and the processing through appropriate pathway. High end-point titers of anti-PA IgG antibodies were maintained until 22 weeks. These were paralleled by high avidity toxin neutralizing antibodies (TNA). Additionally, effective cellular adaptive immunity was generated in the systemic compartment. A significant correlation was found between survival percentage and post-challenge anti-PA titers (r=0.854) and TNA titers (r=0.895). Overall, immune kinetics pointed that differential processing through various compartments gave rise to qualitative differences in the immune response generated by various chimeras.

RESULTS

Construction and expression of various PA expressing chimeras. The structural gene for PA63 was cloned in DNA plasmids bearing the address tags. Authenticity of the DNA constructs was evaluated by transient transfection in J774A.1 mouse MΦ-like cells followed by subcellular fractionation and immunoblot analysis. (Fig.1). Lysosomal fractions were isolated (identified by β-hexosaminidase activity; as shown in *Fig.9, Supplementary Information; SI*) to check for lysosomal localization of PA63 on account of the attachment of a C-terminal LAMP1 address tag. Along with that, cell-culture supernatants were harvested to determine the cellular-secretion of PA63 by TPA leader. SDS-PAGE followed by western blot analysis of the proteins from total cell lysate, lysosome and cell-culture supernatant fractions indicated that PA63 protein along with the targeting signals was being properly recognized by the mammalian cells. As expected, the construct pTPA-PA63 encoded a cell-secreted version of PA63. Whereas, the chimera pPA63-LAMP1, expressed a lysosome-associated form of PA63. However, the chimera pTPA-PA63-LAMP1, encoded a lysosome-associated form of PA63 in addition to a cell-secreted version. The construct pUQ-PA63 solely encoded a cell-associated form. Furthermore, the pQE30 expression plasmid (T7 expression system, Qiagen) bearing the structural gene for PA63, expressed recombinant PA63 (rPA63) protein to homogeneity. Purified rPA63 mediated its function as the receptor binding moiety of anthrax toxin complex in a manner similar to that displayed by full-length rPA83 protein (See *SI, for Fig.10*)

DNA chimeras induced a strong and long lasting systemic anti-PA antibody response. To address the issue whether the targeting signals could deliver the DNA encoded antigen to the immune system and induce an efficient humoral response was assessed. Mice were immunized with DNA chimeras and were bled at 4 week intervals for over a period of 22 weeks, and sera were analyzed for the presence of anti-PA IgG1, IgG2a, IgG2b, and IgA antibodies by ELISA. Following i.m. administration, 100% of the mice developed significant IgG1 antibodies after the first booster dose ($p<0.05$, Fig.2), however, pronounced titers developed only after the second booster dose ($p<0.01$, Fig.2). Significantly higher anti-PA IgG1 titers (≈40,000) were observed following immunization with the construct encoding pTPA-PA63-LAMP1 (Protein boost) as compared to the native construct immunized group ($p<0.001$, Fig.2) and; other groups immunized with constructs bearing the address tags ($p<0.05$). Notably, pTPA-PA63 construct also generated significant titers (≈30,000), following a protein boost, as compared to the native group ($p<0.001$) and other groups that received constructs bearing the targeting signals ($p<0.05$). Mice immunized with pUQ-PA63, pTPA-PA63-LAMP1+pUQ-PA63, pLAMP1-PA63, pTPA-PA63+pUQ-PA63 and native constructs; generated moderate to high IgG1 anti-PA titers. Evaluation of the anti-PA IgA end point titers displayed no significant differences between various groups however; titers $\geq 10^3$ were maintained for over a period of 22 weeks (see *SI Fig.11*). Furthermore, evaluation of the titers of complement fixing antibody i.e. IgG2a, depicted that titers of $\geq 10^4$ developed in the group

immunized with pTPA-PA63, pTPA-PA63-LAMP1 and pUQ-PA63 after the second boost (Fig.3). For rest of the groups the titers ranged between 10^3 and 10^4. A similar trend was also seen for anti-PA IgG2b antibody which also displayed titers ranging between 10^3 and 10^4 (see *SI Fig.12*). No anti-PA reactivity was seen in the serum of mice immunized with PBS and corresponding vector (not shown in figure). We also examined the IgG subclass distribution as an indicator of the T-helper-cell subsets (TH1/TH2) induced by the heterologous prime boost strategy. For all the groups that received chimeras bearing TPA, LAMP1 address tags, the IgG1 titers were approximately 1.0 log higher than the IgG2a responses which clearly indicated a TH2 bias. On the contrary, the chimera pUQ-PA63 displayed a TH1 bias on account of a lower IgG1: IgG2a ratio.

Efficient T-helper cell responses were generated after DNA vaccination. As cytokines play an important role in polarization of T-helper cell responses, we quantified the levels of type I (IL-2, IL-12, IFN-γ) and type II (IL-10, IL-4) cytokines secreted by splenocytes from immunized mice (n=5-6 mice per group) after re-stimulation in vitro with rPA63 (Table.2). IFN-γ: IL-10 ratio was taken as an indirect measure to determine the TH1/TH2 bias. The group immunized with pUQ-PA63 displayed a significantly higher IFN-γ: IL-10 ratio both after a DNA (17.42) and a protein boost (16.27) as compared to all other groups ($p<0.001$, Table.2). On the contrary the IFN-γ: IL-10 ratio for the group pTPA-PA63-LAMP1 was quite lower, 4.5 (Protein boost) and 5.11 (DNA boost). Another group, pTPA-PA63 also displayed significantly lower IFN-γ: IL-10 ratio (1.15 for DNA boost and 2.29 following protein boost1) as compared to the group pUQ-PA63. It was noteworthy, when mice were immunized with a combination of constructs pTPA-PA63 +pUQ-PA63, we observed a significantly higher IFN-γ: IL-10 ratio than when they were immunized with pTPA-PA63 alone (10.3 vs. 1.15 & 2.29, $p<0.01$). The construct pTPA-PA63-LAMP1+pUQ-PA63 also displayed a higher IFN-γ: IL-10 ratio as compared to pTPA-PA63-LAMP1 alone, however, the difference was not significant. The results therefore, depicted that TPA and LAMP1 signals biased the response towards TH2 type however; the bias was much more pronounced when these two signals were combined together as N- and C-terminal address tags. Contrarily, immunization with UQ bearing construct, alone or in combination, resulted in a preferential bias towards a TH1 type immune response.

Efficient B- and T-cell recall responses were generated in vitro. To measure the cellular immune responses elicited by DNA vaccination, splenocytes from immunized mice were isolated to determine PA-specific IFN-γ and IL-4 responses upon re-stimulation with rPA63 in vitro by ELISPOT (Fig.4A & B). Data shows that compared to the control group (immunized with vector and PBS) all other groups developed discernible PA-specific IFN-γ and IL-4 responses. We also included unstimulated and ConA (1.0μg/ml) stimulated splenocytes for all vaccination groups as negative and positive controls, respectively (not shown in figure). The level of SFU/10^6 splenocytes for the ConA stimulated splenocytes was $\approx 10^3$ whereas the number of SFU/10^6 splenocytes for unstimulated controls ranged between 0 and 5 and were not significantly different between the groups. The magnitude of IFN-γ secreting spleen cells for the groups that received pPA63-LAMP1, pTPA-PA63 (DNA boost) and pUQ-PA63 was found to be $\approx$200 SFU/10^6 splenocytes which was quite significant as compared to the group pPA63-Native ($p<0.01$, Fig.4A). Also, the groups that received pTPA-PA63 (Protein boost), pTPA-PA63-LAMP1 (DNA boost) and its combination with pUQ-PA63, or else s.c. injections of rPA63 and rPA83 proteins, generated a significant number of IFN-γ SFUs; $\approx$250 SFU/10^6 ($p<0.001$, Fig 4A). However, the group pTPA-PA63-LAMP1 (Protein boost) displayed the highest IFN-γ SFU/10^6 of ≥250 ($p<0.001$ vs. pPA63-Native, Fig.4A).

We also quantified the PA-specific IL-4 secreting cells after vaccination. As can be seen in the Fig. 4B, the number of IL-4 secreting cells was significantly higher (≥400 SFU/10^6) in the group that received pTPA-PA63-LAMP1 than the group that received pPA63-Native construct ($p<0.001$, Fig.4B). Following closely were the groups that received s.c. rPA63 and rPA83 proteins (≤400 SFU/10^6). Rest of the groups that received TPA, LAMP1 bearing constructs along with their combinations with pUQ-PA63 developed ≤350 SFU/10^6 splenocytes. Overall, the group pTPA-PA63-LAMP1 (both DNA and protein boost) again displayed the highest SFU/10^6 level of ≥400 whereas the group pUQ-PA63 elicited the lowest count of IL-4 secreting cells (≥100 SFU/10^6). Of note, TPA, LAMP1 bearing chimeras generated an overall higher count of IL-4 secreting cells as compared to IFN-γ secreting cells (Fig.4.A & B). However, their combinations with pUQ-PA63 elicited an overall lower frequency of IL-4 SFUs as compared to when they were administered alone.

We also evaluated the number of IgG and IgA secreting cells in the total splenocyte population (Fig.5A & B). Enumeration of the IgG ASCs revealed that the results were in parallel with those obtained for IL-4 secreting cells. The group pTPA-PA63-LAMP1 elicited significantly higher number of IgG (≥600 ASC/10^6) antibody secreting cells as compared to the group that received native PA63 encoding chimera ($p<0.001$, Fig.5A). Following closely were the groups that received recombinant protein immunizations which also elicited a significant count of ≈600 ASC/10^6 ($p<0.001$) as compared to the native group. Evaluation of the IgA ASCs detailed that the frequency of IgA ASCs for most of the groups was ≈300 IgA ASC/10^6 which was quite significant as compared to the pPA63-Native group. The results, therefore, pointed that LAMP1 and TPA address tags favor efficient recall B-cell responses upon re-stimulation with PA in vitro.

Induction of potent cellular proliferation responses on DNA chimera delivery. Spleen cells from the immunized mice were harvested 4 weeks after the last immunization and were re-stimulated for 3 days in the presence of for rPA63 (10μg/ml) for a standard MTT-based in vitro lymphocyte proliferation assay. As shown in Fig.6, pTPA-PA63-LAMP1 DNA construct displayed the highest proliferation index of 15.0 followed closely by the group that received pTPA-PA63 protein boost (PI ≈13). These PI values were quite significant as compared to the group pPA63-Native ($p<0.01$, Fig.6). Of note, the group pTPA-PA63-LAMP1+pUQ-PA63 also elicited a high PI ≈11.0 which was quite significant as compared to the native group ($p<0.05$, Fig.6). Other groups, namely pTPA-PA63 (DNA boost), pPA63-LAMP1, and the ones immunized with recombinant proteins also displayed a similar PI ≈11.0. Rest of the groups that received DNA constructs encoding PA with a UQ targeting signal alone or in combination also mounted a significant PI ≤ 10. The results indicated that almost all the groups mounted strong proliferative responses indicating successful clonal expansion of PA-specific splenic cells on re-stimulation.

Generation of potent cytotoxic T-lymphocyte responses following vaccination. In order to determine the contribution of specific T-cell populations to the observed CTL activity, after 6-day co-culturing of the stimulator cells and nylon wool enriched T cells derived from DNA immunized mice, the $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ cells were separated by a magnetic cell sorting method and tested for their individual abilities to lyse PA peptide-pulsed target cells. Results as depicted in Fig.7. indicate that $CD3^+$ $CD8^+$ T-cells elicited highest cell-cytotoxic potential in the group immunized with pUQ-PA63. These cells efficiently lysed peptide-pulsed target cells but did not lyse normal J774A.1 cells. On the contrary, $CD3^+$ $CD4^+$ T cells isolated from the groups pTPA-PA63-LAMP1 and pTPA-PA63 exhibited highest cell cytotoxic potential. Following closely were the groups immunized with rPA63 and rPA83 that also displayed predominantly $CD3^+$ $CD4^+$ T-cell mediated cytotoxicity. In contrast to the $CD3^+$ $CD4^+$ T cells, $CD3^+$ $CD8^+$ T cells isolated from these groups lysed significantly fewer peptide-pulsed target cells than did $CD3^+$ $CD4^+$ T cells. The groups that received combinations of pUQ-PA63 with LAMP1 and TPA bearing chimeras elicited both, $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T-cell cytotoxic responses however, the lytic ability of the former was higher than the latter. Overall, $CD4^+$ T-cell cytotoxicity was displayed by the groups immunized with chimeras bearing TPA, LAMP1 address tags whereas predominantly $CD8^+$ T-cell cytotoxicity was displayed by groups receiving UQ-bearing chimera.

High avidity toxin neutralizing antibody responses developed following DNA vaccination. Pre-challenge lethal toxin neutralizing antibody (TNA) titers were measured in the serum of all animals 2 weeks after each immunization. Neutralization activity was expressed as percentage survival of the J774.A1 MΦ-like cells following lethal toxin shock in the presence of anti-PA antibodies from the serum of immunized mice. For evaluation of TNA titers, we initially used PA and LF at a concentration of 1.0 μg/ml each. At this concentration the maximal pre-challenge TNA titers obtained for almost all the groups were ≤50 and we could not predict any significant differences in the TNA titers elicited by various groups (See *SI, Fig.13*). The concentration of PA and LF utilized for this assay varies widely, with ranges 0.05 to 1.0μg/ml for PA and 0.01 to 1.0 μg/ml for LF [53-56]. It has, however, been reported that a final concentration of 187.5ng/ml of PA and LF is 4-8 fold more toxin than what is needed to kill 100% of the J774A.1 MΦ-like cells [57]. Therefore, for further evaluation we utilized a concentration 187.5 ng/ml each of PA and LF. Reduction in the concentration of PA and LF revealed the variations in the toxin neutralization response developed by each group. Results also demonstrated that toxin neutralizing activity appeared in

the serum of animals only after the first booster dose (Table 3). These antibodies could efficiently neutralize the lethal toxin action. The group pTPA-PA63-LAMP1 elicited the highest TNA titers of ≥600 for both protein and DNA boost. These titers were significantly higher than the pPA63-Native group ($p<0.001$, Table 3). Groups that received pTPA-PA63 and pPA63-LAMP1 also elicited significant TNA titers ranging from 350-450 ($p<0.001$ and $p<0.01$, respectively, vs. pPA63-Native). Contrarily, the group pUQ-PA63 elicited the lowest TNA titers of ≈100 indicating low toxin neutralizing response. The chimeras, pTPA-PA63 and pTPA-PA63-LAMP1 elicited significantly higher TNA titers as compared to their combinations with pUQ-PA63 ($p<0.05$, Table3). Sera from control mice did not neutralize anthrax lethal toxin at all. Overall the results indicated that TNA titers were significantly higher in the serum of animals immunized with chimeras bearing both the TPA and LAMP1 address tags than the animals immunized with the chimeras bearing them independently or lacking them.

We further examined the avidity of anti-PA antibodies to estimate their ability to neutralize lethal toxin action. We performed a urea based ELISA as mentioned in the materials and methods. Antibodies with high avidity were elicited at week 12 (Table 4). However, 2 weeks following anthrax lethal toxin challenge at week 14, there was a further rise in the avidity of the antibodies for all the groups. Although highest avidities were shown by the groups that received constructs bearing TPA, LAMP1 address tags (AI ranging from 65 to 90), their combinations with UQ bearing chimera also elicited increased avidities (AI≥45) indicating affinity maturation.

Post-challenge anti-PA and TNA titers correlated well with survival against challenge. Immunized mice were challenged with a Letx mixture (50μg PA and 22 μg LF, ≈4-5 LD_{50} of Letx) in a total volume of 100 μl via tail vein injection at different time intervals (week 10, 12, 14 and 16) post last immunization. Kaplan-Meier curves for survival of the DNA vaccinated mice against Letx challenge are summarized in Fig.8. Control mice (PBS and vector immunized) died after receiving a lethal toxin injection with an average MTTD (Mean Time To Death) around 0.5 ± 0.2 days. Analysis of survival curves indicated that the survival percentage varied at all these time points. Highest survival was elicited by all groups when they were challenged at week 12 and 14. A significant correlation was found between survival percentage and post-challenge anti-PA titers and TNA titers which also displayed their peaks at these two time points ($p<0.001$, Table 5).

Statistical differences between the delays in time to death as measured by log-rank statistic, for the groups immunized with TPA and LAMP1 bearing constructs compared to the native construct receiving group were found to be quite significant ($p<0.01$). Time-to-death analysis revealed that DNA vaccination with constructs pTPA-PA63, pPA63-LAMP1and pTPA-PA63-LAMP1 was more protective than the native PA encoding construct. Analysis of the survival percentage displayed by various groups immunized with chimeras bearing the address tags depicted that the group pTPA-PA63-LAMP1 displayed the highest survival frequency of 88% at week 12 and 14 with average MTTD of 8.0±1.5 days. This value was significantly higher than the native group with respect to both survival percentage ($p<0.001$) as well as MTTD ($p<0.01$). Of note, the average anti-PA and TNA titers for this group at these two time points corresponded to $\approx 10^5$ and $\approx 10^3$ (Table 5). Other groups that received pTPA-PA63 (Protein boost) and pPA63-LAMP1 (both DNA and protein boost) displayed an average survival percentage of 63-75% with average anti-PA ranging between 65,000-88,000 and TNA titers between 400-800. The animals that died showed a MTTD of 5.0±1.0 and 4.0±1.5 days for TPA and LAMP1 bearing chimeras, respectively. However, UQ bearing chimeras displayed lowest survival frequency of 38% at these two time points with average MTTD of 5.0±1.0 days. The corresponding value for anti-PA titers and TNA titers for this group was ≈35,000 and ≈250 respectively. Additionally, the groups that received rPA83 and rPA63 also displayed a high survival frequency of 75% with average values of anti-PA and TNA titers of ≥80,000 and ≈800, respectively. Rest of the groups displayed a survival frequency of 50-65 % developed anti-PA titers of ≥38,000 and TNA titers ≥350. However, lowest survival percentage of 35% was displayed by the chimera pPA63-Native which developed anti-PA and TNA titers of ≈20,000 and ≈200, respectively. The results therefore, implied that anti-PA and TNA titers of $\approx 10^5$ and $\approx 10^3$, respectively, provided protection to almost 90% of the animals. Although the chimeras bearing only TPA and LAMP1 signal elicited a high survival percentage, the percentage survival was highest when they were combined together in the chimera pTPA-PA63-LAMP1. Further, when the survival data for all the groups were combined, a significant correlation was found with the protection

conferred by the post-challenge anti-PA and TNA titers ($p<0.001$). Correlation analysis showed that survival at week 12 and 14 correlated well with the post-challenge anti-PA (r=0.854) and TNA titers (r=0.895). Although significant, MTTD ($p<0.01$, r=0.375) could not be correlated with survival.

DISCUSSION

Major drawbacks associated with the currently licensed anthrax vaccines potentiate the need for development of a vaccine that is beset with fewer side effects and can elicit protective immunity using a shorter dosage schedule in response to a bioterrorism event. In this context, there is a broad consensus that DNA vaccines can serve as an attractive target. The ability of DNA vaccines to provide effective immunological protection against infection rests on the generation of efficient $CD4^+$ and $CD8^+$ T cell responses which requires presentation of the vaccine-encoded antigen in context with MHC I and MHC II molecules, respectively. These molecules engage different antigen-processing pathways namely, cytosolic for MHC I and endosomal for MHC II molecules.

It is, therefore, likely that the outcome of an immune response can be influenced by the form of antigen administered and its ability to access various Ag-processing pathways. This would in turn be revealed by the spectrum of B- and T-cell responses represented by initial clonal outburst, polarization of T-helper cell responses (TH1/TH2), antibody isotype switch and B- and T-cell memory responses. Keeping this information in mind, we addressed the question whether targeting the protease-cleaved fragment of protective antigen (PA63) to various subcellular locations with a set of signal sequences could influence the outcome of an immune response in DNA vaccinated mice. Our results demonstrated that it does indeed influence the immune outcome as manifested by the induction of high end point anti-PA titers, high-avidity toxin neutralizing antibodies, and potent cellular proliferative and cytotoxic T lymphocyte responses.

Since the heightening of immune responses appeared to be rooted in the differences in the targeting signals attached, we first addressed the authenticity of the DNA chimeras and the ability of the address tags to target the encoded Ag to desired subcellular locations by transfection analysis followed by subcellular fractionation and immunoblotting. Results indicated that the PA63 protein along with the targeting signals was being properly recognized by the mammalian cells. LAMP1 signal successfully mediated the lysosomal targeting of PA63. On the other hand, TPA signal mediated the secretion of PA63 into the cell-culture supernatants. Combining these two signals lead to the lysosomal localization as well as cell-secretion of PA63. UQ signal however, solely encoded a cell-associated form of PA63. Hence, all the address tags successfully mediated the targeting of PA63 to various sub-cellular locations aimed at. Regardless of the signal sequence attached, all the constructs showed comparable expression levels.

Following that it was most important to examine the nature of anti-PA IgG antibody subclass induced by DNA vaccination. The pattern of IgG subclass is known to mirror the T-helper cell-derived cytokine responses [58] and different IgG subclasses (i.e. the complement fixing IgG2a Abs vs. non-complement fixing IgG1 and IgG2b) are involved in distinct mechanism of host protection [59]. All these facts argue for detection of anti-PA IgG subclass pattern to predict the correlates of protective immunity. Analysis of the end-point titers of IgG1 antibody indicated that titers of highest magnitude were elicited by the group immunized with pTPA-PA63-LAMP1 (both DNA and protein boost). Not only that, this group also mounted high end-point titers of complement fixing IgG2a antibody. Close examination of IgG subclass distribution indicated a higher IgG1:IgG2a ratio whenever the groups were immunized with chimeras bearing TPA, LAMP1 signals alone or when they were combined together in a chimera as N- and C-terminal address tags. Importantly, electron microscopic studies have shown that spore-associated proteins can be recognized by anti-PA antibodies, and PA-immune serum from several species enhanced the phagocytosis of spores of the virulent Ames and Sterne strains by murine peritoneal macrophages [60, 61]. In addition, it has been shown that IgG1 subclass displays highest affinity towards PA [62]. Therefore, development of high end-point titers of IgG1 antibody upon DNA vaccination gains high merit from anti-spore activity point of view. It is also noteworthy that these high end-point titers were maintained until 22 weeks. Maintenance of such high end-point anti-PA titers can aid in controlling pathogen spread and potentially in reducing disease progression. Thus, high end-point titers of anti-PA antibodies arising as a result of targeted DNA vaccination can overall act to, (1) enhance sporicidal activities of macrophages [60, 61]; (2) impede spore

germination in vivo [62,63] and; (3) enhance the ability of the early responding cells to ablate infection [63].

There also exists compelling evidence that toxin neutralizing antibodies afford protective immunity [64-66]. The functional significance of the development of high TNA titers gains considerable importance in light of the fact that death of the infected animal occurs due to accumulation of lethal level of toxin although the *Bacillus* itself is sensitive to anti-bacterials [67]. Therefore, we assayed the pre-challenge TNA titers to predict any correlation between the presence of toxin neutralizing antibodies in the serum and protection against lethal challenge. However, analysis of TNA titers emphasized that the conditions utilized in the performance of toxin neutralization assay, like the concentration of PA and LF, are very important for the ability of the assay to correlate with protection in vivo. No significant titers could be detected when a high Letx dose (1.0 μg/ml each of PA and LF) was taken. Evaluation of TNA titers in the presence of 187.5ng/ml of Letx (4-8 fold toxin dose) revealed the variations in the titers elicited by different vaccination groups. Various other studies have also pointed the importance of the assay conditions, like, prior incubation of toxin with anti-PA serum before subjecting the MΦ cells to a lethal shock [30, 31, 35], and passive transfer of antibody before injecting Letx [57, 68]; to determine toxin neutralizing activity in vitro and in vivo, respectively. Most importantly, high toxin neutralizing activity appeared in the serum of the immunized animals after the first booster dose which was paralleled by the generation of high avidity antibodies. Further rise in avidity of the antibodies towards PA, post-challenge, depicted affinity maturation. Since high avidity antibodies are conducive for rapid neutralization of anthrax toxin, these antibodies can well act against the toxin secreted by *Bacillus anthracis*. Taken together, these Letx neutralization antibodies and anti-PA antibodies generated upon immunization with various DNA chimeras can provide a parallel line of defense against the toxemia as well as bacteremia.

Immunization studies with AVA (US anthrax vaccine) have demonstrated a decreasing serum anti-PA antibody titer in humans over time [69]. Also a gradual decline in antibody titer over time, a parameter that is currently being used to determine the immunological status after vaccination, argues for periodic booster inoculations to maintain an appreciable titer. Thus, many variables are evaluated to predict the correlates of protective immunity, for e.g., persistence of circulating antibody post-immunization, antibody titers generated in surviving mice post-challenge and the time at which peak titers are attained or the time elapsed before the titers return to baseline. To this end, we assessed the anti-PA antibody response, in terms of total anti-PA and TNA titers prevailing in vaccinated animals post-challenge that aided protection. Results detailed that anti-PA and TNA titers were maintained until week 18 (Table.5). Comparison of the antibody titers between various groups indicated that the chimeras bearing the address tags TPA, LAMP1 or both, mounted pronounced anti-PA and TNA titers. Changes in the survival of the immunized mice with regard to anti-PA and TNA titers revealed that titers at week 12 and 14 were quite significant ($p<0.001$, Table5). Anti-PA titers of $\approx 10^5$ and TNA titers of $\approx 10^3$ provided protection to ≈90% of the vaccinated animals. In certain studies with guinea pigs [64, 70] and rabbits [65] it has been shown that anti-PA titers of ≥300 and $>10^5$ respectively, confer 100% protection. Thus far, the minimum protective anti-PA antibody titers differ depending upon variables of the host [71].

Further analysis demonstrated that quantitative anti-PA and TNA titers at week 12 and 14, in addition to displaying their peaks at these two time points, were significant predictors of survival. Correlation analysis showed that survival at week 12 and 14 correlated well with the post-challenge anti-PA ($r=0.854$) and TNA titers ($r=0.895$). Although significant, the mean-time-to-death ($p<0.001$, $r=0.375$) could not be correlated with survival at these two time points. In previous studies with AVA immunized NZW rabbits, anti-PA and TNA titers at week 6, 8 and 10 were found to be significant predictors of immunity [72, 73]. Likewise, another study with guinea pigs indicated that the anti-PA and TNA titers at the time of challenge (week 6) were of predictive value in evaluating survival [64]. Possible factors contributing to differences between earlier studies and ours may be the form of antigen (DNA vs. protein) or else the animal model (mice vs. rabbits and guinea pigs). Our conclusions also receive support from earlier observations that disease pathogenesis or intrinsic antibody titers differ with respect to host [64, 65, 70, 71] and thus, contribute to the variable predictors of protective immunity.

Although the production of antibody is necessary for protection against anthrax infection, it is recognized that a cell-mediated component is also required [29, 74]. Furthermore and consistent with this observation is the well-documented fact that effective

cellular adaptive immunity requires the co-stimulatory signals and cytokines released by antigen-activated T-helper subsets [75]. In this regard, we evaluated the cytokine secretion profile of the T-helper cell subsets arising as a result of DNA vaccination. Our results clearly indicated that differential targeting was responsible for T-helper cell differentiation. Dissection of the cytokine secretion pattern indicated the generation of primarily TH2 biased responses on account of the attachment of the signals LAMP1, TPA or both (as measured indirectly from the IFN-γ: IL-10 ratio). Contrarily, inclusion of UQ signal in the DNA chimera resulted in a preferential bias towards TH1-type of immune response. Characterization of PA-specific effector T-cell responses (enumeration of cytokine secreting cells and T-cell mediated cytotoxicity) indicated the generation of effective cellular adaptive immunity in the systemic compartment. These results are very significant in light of recent studies that have pointed the role of IFN-γ producing $CD4^+$ T lymphocytes in protection against virulent challenge [76]. Overall, the idea to target PA to both endocytic and cytosolic compartments was successfully exploited to generate efficient effector T-cell responses.

However, our findings are quite striking in view of the fact that all the vaccine combinations (5 out of the 7 tested) bearing the signals LAMP1 and TPA alone or together, aided the generation of TH2 biased responses as indicated by indirect IFN-γ: IL-4 and IgG1:IgG2a ratios. Further characterization of PA-specific effector B-cells indicated potential plasma IgA and IgG secretion. DNA vaccines have long been known for their capability to elicit TH1 biased responses favoring potent cytotoxic T-lymphocyte responses and weak in their ability to generate efficient humoral immunity [77, 78]. However, our study demonstrates high levels of plasma IgG and IgA secretion along with the generation of effective cellular adaptive immunity. In addition these responses were found to be at par with recombinant PA63/83 vaccinations (formulated with aluminum hydroxide adjuvant). Of note, anthrax is primarily a systemic disease resulting from the release of anthrax toxin in peripheral lymph nodes and in the general circulation [2, 79]. Therefore, development of effective cellular and humoral immunity in the systemic compartment is of immense importance.

How do these signals mediate such a large impact on the immune outcome-was the next question we addressed? Analysis of the capacity of the processing compartments to generate peptides threw some light on this aspect. Processing of antigens can occur potentially through two pathways i.e., proteasomal degradation of the endogenous antigen and TAP transport to ER followed by binding to MHC I [52] or else degradation and loading of the exogenous antigen within the endosomes leading to MHC II loading [44, 45]. Furthermore, within the endosomal pathway different endocytic compartments are involved in generating different peptides [80]. The LAMP1 protein resides mainly in lysosomes with minor amounts present in late endosomes as well. These compartments are defined by the differences in the accessibility to different endocytic tracers and pH [81]. Differential processing in these compartments can therefore, give rise to both qualitative and quantitative differences in the generation of peptides from these compartments [82]. That probably explains why we observed differences in the magnitude and type of immune responses generated by the chimeras bearing TPA and LAMP1 signals although both of them targeted the antigen to endosomal pathway.

Of note, it is not only the targeting of the antigen to a pathway that results in an effective immune response but the generation of specific epitopes that confer protective immunity [68, 83]. This probably was attained by attaching the signal sequences, LAMP1 and TPA, as N- or C-terminal address tags. Naturally, PA is secreted during an infection and undergoes structural rearrangements inside the endosome triggered by the low pH in this compartment [2, 12, 79]. Therefore, targeting of PA63 to endosomal/ lysosomal compartments by DNA vaccination on account of the attachment of TPA and LAMP1 signals, alone or in combination, probably came closer to the generation of antigenic determinants that were needed for the generation of a better T-helper, cytotoxic and neutralizing antibody response. Our observation that generation of optimal epitopes is an absolute necessity to confer protective immunity receives support from earlier studies that have shown that monoclonal antibodies corresponding to specific epitopes are protective against anthrax lethal challenge [84]. However, generation of a response of highest magnitude, when these two signals were combined together, can be attributed to the attainment of a synergism in giving rise to optimal, specific, protective epitope peptides from both the endosomal/lysosomal compartments. This synergism was probably not attained when these two signals were present singly.

Inability of the UQ bearing chimera to generate protective immunity alone or in combination with TPA

and LAMP1 bearing constructs could be as a result of the lack of optimal epitope generation on account of enhanced proteasomal degradation. Reports suggest that sustained protein expression may lead to an increased antigenic load and enhanced immunogenicity [85]. Although rapid proteasomal degradation of PA should lead to enhanced presentation by MHC I molecules, it may prevent the protein to reach the extracellular space and thus the APC surrounding the infectious site. In addition, the heightening or depreciation of an immune response is very likely dependent upon the dynamic relationship between the encoded antigen and the immune system. For maximal effect the antigenic epitopes derived must be presented to the immune system at a certain rate, concentration and order [86]. It is possible that the lack of stability of the PA63 protein expressed by pUQ-PA63 prevented any kind of synergism, in giving rise to optimal epitopes with those arising from TPA and LAMP1 bearing chimeras to generate an additive effect. However, the role of protein stability vs. immune response generation would require further analysis to evaluate its overall impact. Overall, all these factors contributed to the generation of heightened responses for the groups bearing TPA, LAMP1 signals as compared to the one bearing UQ signal.

Apart from this, the combinations that received protein boosters also mediated heightened responses as compared to the groups that received a DNA booster. Prime-boost (priming with DNA and boosting with protein) has been accredited to be a well known strategy for boosting immune responses [35, 40-42, 87]. However, the mechanism by which a protein booster amplifies CTL or antibody responses is relatively unclear. Increased antigen availability at the crucial stage of boosting might be an explanation. It is known that for antibody induction, in addition to an effective T-cell help, more available antigen is provided by boosting for uptake by B cells, a factor favoring B-cell activation [88, 89]. Time taken for antigen expression followed by its uptake by B cells can, therefore, be a crucial parameter in boosting immune responses. That might explain why boosting with protein was more effective at raising specific antibody levels than DNA alone. However, a more striking result was evident in our prime-boost setting, i.e. the chimera pTPA-PA63-LAMP1 afforded equivalent protection both after DNA or a protein boost (88%) which indicated that the protective effect was maximal when the two signal sequences, TPA and LAMP1, were combined together as N- and C-terminal tags. Thus, the results pointed that immune outcome showed a greater dependence on targeting of the antigen to appropriate cellular compartment (s) rather than on prime-boost regimen.

Another aspect that was intriguing was the surprising better performance of our DNA chimera pTPA-PA63-LAMP1 over s.c. recombinant protein vaccine preparations (rPA63/83 aluminum hydroxide formulation). While it is very much established that recombinant protein vaccine preparations fare better than DNA vaccine preparations, their combinations with various adjuvants can stimulate diverse immune responses. Studies have shown that guinea pigs inoculated with rPA/Ribi adjuvant were completely protected against a lethal challenge but were poorly protected when vaccinated with either rPA/alhydrogel or the licensed UK human anthrax vaccine [29] which is an aluminum hydroxide adsorbed supernatant material preparation (mainly PA) from fermentor cultures of toxigenic, non-encapsulated isolate of *B.anthracis* V770-NP1-R. Also, it was found difficult to protect guinea pigs by vaccination with aluminum-containing human vaccines [27]. Thus, the vaccine-adjuvant combinations may or may not confer protection and these responses further vary between species [90].

Thus far, clinical studies of anthrax vaccine evaluation have relied only on determination of seroconversion and the contribution of the cellular adaptive immunity has not been thoroughly investigated. However, a recent study has suggested the role of IFN-γ producing $CD4^+$ T lymphocytes in protection against anthrax [76]. Importantly, such a protective IFN-γ pathway might only be functional when Letx is absent or neutralized by anti-toxin antibodies as Letx has been shown to inhibit $CD4^+$ T lymphocyte activation by disrupting antigen-receptor signaling [16, 91, 92]. In such a scenario the generation and maintenance of high end-point anti-PA titers and TNA titers alongwith the generation of effective cellular adaptive immunity in the systemic compartment can provide a parallel line of defense against the pathogen as well as the toxin and embody the paradigm of antibacterial and antitoxic components in a DNA vaccine preparation. Thus, we envision that the pTPA-PA63-LAMP1 can represent a prototypic, safe and efficacious DNA chimera for further evaluation and development of DNA vaccine against anthrax.

MATERIALS AND METHODS

DNA vaccine plasmids and recombinant proteins. DNA manipulations were performed according to standard molecular biology procedures [93] using the *E. coli* DH5α strain. Structural gene of protease-cleaved fragment of protective antigen (PA63) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using sequence-specific primers (For primer sequences see *SI Table. 6*) and cloned in eukaryotic plasmids bearing address tags. The sequences of clones bearing the address tags, pPA63-Native: (GenBank accession no. EU249810), pTPA-PA63 (GenBank accession no. EU249808), pUQ-PA63 (GenBank accession no. EU249809), pPA63-LAMP1 (GenBank accession no. EU249806), pTPA-PA63-LAMP1 (GenBank accession no. EU249807) were confirmed by sequencing. Similarly, the structural gene for protective antigen (1.6 Kb) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using sequence-primers (*see SI Table. 6* for primer sequences) and cloned in pQE30 expression vector. rPA63 was expressed as a fusion protein with 6x histidine tag in *E.coli* M15 strain and was purified on a Ni^{2+}- NTA column to more than 95% homogeneity from inclusion bodies as described previously [94]. An additional purification step was performed to remove endotoxin contaminations (lipopolysaccharide) using a procedure described previously [95]. The protein was eluted with a gradient of 0 to 1 M NaCl in $T_{10}E_5$. The rPA 63 was dialyzed against 10 mM HEPES overnight and stored in aliquots at -70°C. Full-length rPA83 protein was also expressed and purified using the same procedure

Plasmid DNA preparation. Plasmid DNA was prepared from overnight cultures of transformed DH5α bacteria in Luria Bertani Broth (Amersham) plus 50 μg/ml kanamycin sulfate (Amersham) and processed by using Endo-free Giga kits (Qiagen, Valencia, CA).

Transfection and western blot analysis. J774.A1 mouse MΦ-like cells were seeded at a concentration of 2-3 $\times 10^7$ cells into a 75-cm^2 flask (Corning costar) until the cells reached approximately 50–70% confluence. Plasmid DNA transfection was performed with LipofectAMINE 2000 (Invitrogen) reagent, as specified by the manufacturer. Transfected J774A.1 cells were washed in ice-cold Hanks buffered salt solution (HBSS), gently scraped into fresh HBSS (10 ml) and centrifuged at 145g for min. The pellet was washed in homogenization buffer, pH 7.0 (4°C, 0.25M sucrose; 20mM HEPES; 0.5Mm EDTA), re-centrifuged as above and finally resuspended in fresh homogenization buffer (1.5 ml). Whole cells were ruptured as described previously [96] at a concentration of 1.5-2.0 $\times 10^7$ cells/ml. The ruptured cells were centrifuged at 800g for 10 min at 4°C to remove unbroken cells and nuclei.. Ficoll (Pharmacia) and Nycodenz (Nycomed) density gradients were prepared as described earlier [97]. The cell supernatant was layered on the top of the gradient and the sealed tube was centrifuged in Beckman VTi 65.2 rotor at 240,000g and 4°C for 90 min, using slow acceleration and deceleration programs. Fractions were collected (26x200μl) from the bottom of the centrifuge tube and stored on ice. The presence of lysosomes in different fractions was determined by analyzing the activity of β-hexosaminidase [98]. The J774A.1 culture supernatant proteins were precipitated by ice-cold acetone. For western blot analysis, the total cells were lysed in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) 36 h post transfection. The proteins from the lysosomal fraction and cell-culture supernatant were solubilized in solubilization buffer (60mM TriS-HCl (pH6.8), 1% β-mercaptoethanol, 1% SDS, 10% Glycerol). Finally, the solubilized proteins from the total cell lysate, lysosomes and cell-culture supernatants were subjected to 12% SDS–polyacrylamide gel electrophoresis under denaturing conditions with 50 mM dithiothreitol (DTT). Proteins from the gel were transferred to a nitrocellulose membrane and probed with rabbit anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG (Amersham biosciences).

Vaccination and lethal toxin challenge. Six- to eight-week-old female Balb/c mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA suspended in phosphate-buffered saline (PBS; 50 μl per hind leg) administered via a 26-gauge, 1-ml hypodermic needle. All mice received the first DNA booster dose 28 days after their initial immunization and received either a second DNA booster (100μg, i.m.) or a s.c. injection of rPA63 (12.5 μg formulation with Incomplete Freunds Adjuvant) 28 days thereafter. Sera were obtained from blood samples collected at 4 wk intervals. For lethal toxin challenge, 50 μg of PA83 and 22μg of LF were injected intravenously via the tail vein, and the mice were closely monitored for 15 days.

ELISA detection of anti-PA reactivity in plasma. Anti-PA reactivity of the sera from immunized mice was determined by direct ELISA. Briefly, microtiter plates were coated with rPA63 (10μg/ml). Following blocking in 5% BSA and washes in PBS-Tween 20 (0.2%), plates were incubated with goat anti-mouse IgG, IgG1, IgG2a, IgG2b and IgA HRP conjugate (Santacruz Biotechnology) for 1 h at 37°C. After washes in PBS-Tween 20 (0.2%), plates were developed using TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

Detection of toxin neutralizing antibody titers (TNA) in plasma. The protective effects of anti-toxin Abs were determined using a previously described assay [57] that measures their capacity to protect the J774A.1 mouse MΦ-like cell line from Letx. Briefly, J774A.1 MΦ cells (5×10^4 cells/well) were seeded in 96-well, flat bottom plates. After the cells reach 50-70% confluency, serum dilutions were added together with Letx (187.5ng/ml each of PA and LF) and incubated for 4h. After an additional incubation of 30 min after the addition of MTT (0.5mg/ml) (Sigma-Aldrich), the cells were lysed with acidic isopropanol (0.04-0.1N HCl in 90% isopropanol). The A_{450} of 100% viable cells was calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

In vitro cytokine production. Splenocytes from the immunized mice were incubated alongwith with rPA63 (10 μg/ml), ConA (1.0 μg/ml; Sigma-Aldrich), or alone in complete medium (RPMI 1640 medium with glutamine supplemented with 10% fetal calf serum, antibiotics, and 5×10^{-5} M β-2-mercaptoethanol) in triplicates in 24-well flat-bottom plates at 37°C, 5% CO_2. Cell culture supernatants were harvested at for 24 hrs post stimulation for IL-2, IL-4 and IL-12 cytokine measurement and, at 72 hrs for IL-10 and IFN-γ measurement. Cytokine-specific sandwich ELISA was performed using OptEIA kit for the specific cytokine (BD Pharmingen) according to manufacturer's protocols.

IFN-γ and IL-4(ELISPOT) assays. IFN-γ and IL-4 responses specific for PA were determined by mouse IFN-γ and IL-4- specific ELISPOT kit (BD Biosciences) as described by manufacturer's protocol. Briefly, multiscreen filtration plates (96 wells; Millipore, France) were coated overnight at 4°C with 4 μg/ml of rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen, San Diego, CA) and rat anti-mouse IL-4 antibody (clone 11 B 11) Thereafter, the plates were washed and blocked with complete medium. Serial twofold dilutions of the spleen cell suspensions from immunized mice were added to the wells starting at a concentration of $5x10^5$ cell per well and re-stimulated in vitro for 48 hrs with rPA63 (10μg/ml), ConA (1.0μg/ml) or in medium alone. After extensive washes, the plates were revealed by incubation with 4.0μg/ml of biotinylated rat anti-mouse IFN-γ antibody (clone R4-6A2; PharMingen) and biotinylated rat anti-mouse IL-4 antibody (clone BVD6-24G2) followed by incubation with streptavidin-alkaline phosphatase (PharMingen). Finally, spots were revealed using BCIP/NBT (Sigma Aldrich) as the substrate. The number of IFN-γ and IL-4-producing cells was determined by counting the number of spot-forming (SFU) in each well with the ELISPOT reader (IMMUNOSPOT, CTL technologies), and the results were expressed as numbers of SFU per 10^6 cells.

Evaluation of antibody-secreting cells (ASC) by the ELISPOT assay. The splenocytes from immunized mice were re-stimulated in vitro as mentioned above. The number of PA-specific ASCs (IgA and IgG) were determined using biotinylated goat anti-mouse IgG or IgA (Sigma-Aldrich) and Alkaline phosphatase-labeled streptavidin-avidin (BD PharMingen) at a dilution of 1/800 in PBS-T following methods described previously [99]. Results were expressed as number of ASC per 10^6 cells.

Lymphocyte proliferation assay. Spleen cells (3×10^5 cells) from the immunized mice were seeded in 96-well tissue culture plates (BD falcon). Cells were incubated with purified rPA63 protein (10μg/ml) and incubated at 37°C in 5% CO_2 atmosphere for 72 h. Cells stimulated with ConA (1μg/ml) and medium alone served as positive and negative controls, respectively. Proliferation was measured by MTT (Sigma-Aldrich) dye based-assay [57]. Proliferation index (PI) was calculated as the ratio

of the average O.D. value of wells containing antigen-stimulated cells to the average O.D. value of wells containing only cells with medium.

Preparation of effector T cells. Peptide-pulsed (synthetic overlapping peptides derived from PA sequence) spleen cells (mitomycinC treated) from Balb/c mice were taken as stimulator cells. Splenocytes from the immunized mice were resuspended in 2.0 ml of complete RPMI containing β-2-mercaptoethanol (5×10^{-5} M) and passed through nylon wool columns to enrich for T lymphocytes as described previously [100] to separate non-adherent T cells. The cell number and cell viability were determined by trypan blue exclusion method. The enriched T cells were resuspended in complete RPMI and seeded in 24 well plates at a concentration of $0.4x10^6$ cells per well. Stimulator cells were also added to the wells at a concentration of $0.4x10^6$ cells per well. The mix of enriched T cells and stimulator cells was then incubated for a period of 6 days at 37°C and 5% CO2. After 6-days coculturing, cell were collected and live effector cells were obtained by removing dead cells by Histopaque (1083) centrifugation as described previously [101].

Magnetic cell sorting. $CD4^+$ and $CD8^+$ T cells were purified by immunomagnetic methods [102]. Briefly, live T cells isolated by Histopaque column purification were incubated with MACS magnetic MicroBeads to which monoclonal antibodies against CD4 molecule (clone GK1.5; isotype, rat IgG2b) or CD8 molecule (clone 53-6.7; isotype, rat IgG2a) had been coupled (Miltenyi Biotec, Calif.) at the concentration of 10 µl of MicroBeads per 10^7 total cells for 15 min in a refrigerator at 4°C. The cells were then washed with PBS supplemented with 2 mM EDTA and 0.5% bovine serum albumin. Following passage of the cells through a steel wool column in a magnetic field, the positively selected $CD4^+$ or $CD8^+$ T cells were eluted out. The purity of the selected $CD4^+$ or $CD8^+$ T-cell population was above 92% as determined by flow cytometry using the appropriate specific monoclonal antibodies as described above.

Preparation of target cells. Peptide-pulsed J774A.1 ($H\text{-}2^d$) mouse MΦ-like cells were taken as target cells. Only J774A.1 cells were also included to check non-specific lysis.

Cytotoxicity assays. The target cells were incubated with effector cells obtained from vaccinated mice at different Effector: Target ratios (5:1, 10:1, and 15:1) for 16 hr. Neutral red uptake assay, a non-radioactive assay procedure [103] was followed to assay CTL activity. The percentage of specific lysis was calculated as (O.D. of control − O.D. of experimental group)/O.D. of control × 100.

Determination of avidity of PA-specific IgG antibodies. Antibody avidity was measured by ELISA using urea as chaotropic agent to dissociate low-affinity antigen-antibody complexes [104]. Briefly, serum samples (in twofold dilutions) were incubated for 1 h at 37°C in ELISA plates coated with rPA63. Plates were washed with PBST and soaked with 4 M urea for 10 min. After subsequent washes with PBST, bound IgG was detected with HRP-linked anti-mouse IgG using TMB as substrate. Endpoint titers for each serum sample were determined as described above, in the presence and absence of urea. Results are expressed as an avidity index, calculated as the percentage of residual activity (endpoint titer) using the following formula: (endpoint titer in the presence of urea)/(endpoint titer in the absence of urea) x 100. Positive and negative controls of known antibody titer (calibrated controls) were run in each assay. During the standardization of the assay, several concentrations of urea (0 to 8 M) were tested, and 4 M was selected as optimal; higher urea concentrations completely abrogated antibody binding.

Statistical analysis. The experimental data were analyzed by software programs Sigma Plot 8.1 or Excel (Microsoft) and were expressed as mean ± SE. The statistical significance of differences was analyzed by a two-tailed Students t-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A *P*-value of <0.05 determined was considered statistically significant. Differences in the survival were evaluated using log-rank analysis of the Kaplan-Meier curves. Correlation coefficients were determined by linear regression analysis.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

FIG.1. The address tags efficiently target PA63 to various sub cellular locations. Shows expression of different forms of protease-cleaved fragment of protective antigen in J774.A1 mouse MΦ-like cells. Cells were transfected with DNA constructs. Cell lysates and lysosomal fractions were prepared 36 hrs post-transfection. Alongwith that cell culture supernatant proteins were harvested by acetone precipitation. Subsequently, the protein samples were subjected to 12% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane. Blot was probed with anti-PA polyclonal serum followed by alkaline phosphatase-conjugated anti-rabbit IgG and developed using BCIP/NBT as substrate.

FIG.2. High anti-PA IgG1 titers were generated by DNA bearing TPA and LAMP1 tags. Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG1 titers elicited following immunization were monitored till 22 weeks. Constructs pTPA-PA63-LAMP1 and pTPA-PA63 generated significantly higher anti-PA IgG1 titers after a protein boost as compared to the group pPA63-Native ($p<0.001$) and; other groups that received chimeras bearing the address tags ($p<0.05$). Each curve represents IgG1 titers elicited by a single mouse representative of 6-7 mice tested in a group in two independent experiments and results are expressed as mean ±SE. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG1 titers.

FIG.3. High anti-PA IgG2a titers were generated by DNA chimera bearing UQ tag. Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG2a titers elicited following

immunization were monitored till 22 weeks. All the groups generated significant IgG2a titers as compared to the control groups that received PBS and vector ($p<0.05$). Each curve represents IgG2a titers elicited by a single mouse representative of 6-7 mice tested in a group in two independent experiments and results are expressed as mean ±SE. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG2a titers.

FIG. 4.A & B. Robust T-cell responses were generated upon restimulation with PA in vitro. Splenocytes from the immunized mice were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 48 h. Figure shows the number of IFN-γ **(A)** and IL-4 **(B)** secreting cells as determined by ELISPOT assay. Each bar represents number of SFU's obtained for a single mouse representative of 4-6 mice tested in a group in three different experiments and results are expressed as mean ±SE. There were significant differences in the number of PA-specific IFN-γ and IL-4 SFUs between the group receiving the native construct and the groups receiving chimeras bearing the address tags. All the p values represent significant differences between the groups that received chimeras bearing the address tags compared to the pPA63-Native group ($p\leq0.001$, **, $p<0.01$ and *, $p<0.05$. The mean number of spots in the presence of medium alone or ConA was ≤5.0 and $\geq10^3$, respectively, and were not different between the groups.

FIG.5. A & B. Enhanced recall B-cell responses were induced by DNA vaccination. Splenocytes from the immunized mice were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 48 h. Figure shows the number of IgG **(A)** and IgA **(B)** secreting cells as determined by ELISPOT assay. Each bar represents number of ASC's obtained for a single mouse representative of 4-6 mice tested in a group in three different experiments and results are expressed as mean ±SE. There were significant differences in the number of PA-specific IgG and IgA ASCs between the group receiving the native construct and the groups receiving chimeras bearing the address tags. All the p values represent significant differences between the groups that received chimeras bearing the address tags compared to the pPA63-Native group ($p\leq0.001$, **, $p<0.01$ and *, $p<0.05$. The mean number of spots in the presence of medium alone or ConA was ≤5.0 and $\geq10^3$, respectively, and were not different between the groups.

FIG.6. Potent proliferative responses were generated by DNA vaccination. Splenocytes from the immunized mice were isolated 4 weeks after last immunization and were re-stimulated with rPA63 (10μg/ml) protein in vitro for 72 h. Proliferation was measured by a MTT (Sigma-Aldrich) dye based assay. The proliferation index (PI) was calculated from the ratio of the average O.D. value of wells containing antigen-stimulated cells to the average O.D. value of wells containing only cells with medium. Each bar represents PI obtained for a single mouse representative of 4-6 mice of a group tested in two different experiments and results are expressed as mean ±SE. There were significant differences in the PI elicited by the group that received pPA63-Native construct and the groups receiving chimeras bearing the address tags. All the p values represent significant differences between the groups that received chimeras bearing the address tags compared to the pPA63-Native group. No proliferation was seen in the un-stimulated controls.

FIG.7. $CD4^+$ T-cell dependent cytotoxicity was generated by DNA chimeras bearing TPA, LAMP1 address tags whereas $CD8^+$ T-cell dependent cytotoxicity was displayed by DNA chimeras bearing UQ address tag. Effector CTLs were assayed for their ability to lyse target cells. Peptide-pulsed J774A.1 ($H\text{-}2^d$) MΦ-like cells were taken as target cells. Normal J774A.1 cells were also included to check non-specific

lysis. Lytic ability of the effector $CD4^+$ and $CD8^+$ CTLs on antigen-pulsed target cells was evaluated at different Effector: Target ratios (5:1, 10:1, and 15:1). Neutral red uptake assay, a non-radioactive assay procedure [101] was followed to assay CTL activity. The percentage of specific lysis was calculated as (O.D. of control − O.D. of experimental group)/O.D. of control × 100. CTL activity of effector CTLs from mice (that received a protein boost) on PA-pulsed target cells is shown by filled circles and on normal J774A.1 cells as open circles. CTL activity of effector CTLs from mice (that received a DNA boost) PA-pulsed target cells is shown by filled triangles and on normal J774A.1 cells only as open triangles. Vector and PBS immunized control mice did not show any CTL activity (not shown in figure). Each curve represents CTL activity displayed by a single mouse representative of 4-6 mice tested in a group in two different experiments and results are expressed as mean ±SE.

FIG.8. DNA immunization provides efficient protection against Letx challenge. The figure shows Kaplan-Meier curves of survival of the immunized mice challenged with a Letx mixture (50μg PA and 22μg LF) by tail vain (i.v.) injection at different time points post immunization (week 12, 14, 16 and18) and the survival percentage was plotted against the Mean time to death (in days). Challenge was 100% fatal in control mice immunized with vector and PBS. Data shows the percent survival of 8-9 mice per group compiled by results from three independent experiments.

Table 1. DNA constructs and the targeting signals attached.

PLASMID NAME	EXPRESSED PRODUCT
pTPA-PA63	N-terminal TPA signal, and 63 kDa mature protein.
pPA63-Native	63 kDa mature protein.
pLAMP1-PA63	C-terminal LAMP1 membrane anchor and 63 kDa mature protein.
pUQ-PA63	N-terminal Ubiquitin leader and 63 kDa mature protein.
pTPA-PA63-LAMP1	N-terminal TPA signal, C-terminal LAMP1membrane anchor and 63kDa mature protein.

Table 2. Cytokine production by spleen cells from the mice immunized with various DNA chimeras.

	CYTOKINE PROFILE [a]					
VACCINE COMBINATION	IL2[b),e)] (pg/ml)	IL12[b),e)] (pg/ml)	IFNγ[c),e)] (pg/ml)	IL4[b),e)] (pg/ml)	IL10[b),e)] (pg/ml)	TH1:TH2[d)]
pTPA-PA63 Pro	52.7 ± 5.5	1162.8 ± 25.5	1079.7 ± 104.1	6.62 ± 1.2	470.5 ±120.4	2.29
pTPA-PA63 DNA	43.3± 6.8	227.1 ± 59.9	189.9 ± 122.1	3.41 ± 0.91	165.5 ± 12.3	1.15
pUQ-PA63 Pro	25.8 ± 5.7	135.2 ± 23.5	1600.2 ± 207.6	3.08 ± 0.87	98.35 ± 45.7	16.27[f)]
pUQ-PA63 DNA	9.72 ± 3.2	150.4 ± 21.6	1296.2 ± 89.7	3.41 ± 0.78	74.44 ±12.9	17.42[f)]
pTPA-PA63-LAMP1 Pro	90.9 ± 15.1	2770.9 ± 169.8	1251.8 ± 334.9	41.6 ± 5.9	277.7 ± 15.5	4.50
pTPA-PA63-LAMP1 DNA	27.8 ± 2.6	1970.9 ± 150.8	1003.12 ± 45.9	40.9 ± 9.0	196.3 ± 35.9	5.11
pPA63-LAMP1 Pro	61.5 ± 11.4	680.0 ± 109.8	177.08 ± 34.9	53.0 ± 12.5	108.3 ± 27.9	1.64
pPA63-LAMP1 DNA	19.8 ± 4.8	780.9 ± 100.8	186.04 ± 56.8	41.0 ± 12.8	135.5 ± 45.1	1.37
pPA63-NATIVE Pro	91.4 ± 19.1	133.8 ± 18.8	294.79 ± 40.9	3.45 ± 0.89	154.1 ± 22.7	1.91
pPA63-NATIVE DNA	18.4 ± 3.9	109.04 ± 25.8	263.54 ± 39.8	3.17 ± 0.99	96.38 ± 17.7	2.73
pTPA-PA63+ pUQ-PA63 Pro	74.5 ± 14.2	102.34 ± 33.8	887.7 ± 58.99	5.4 ± 1.5	85.8 ± 22.9	10.35
pTPA-PA63+ pUQ-PA63 DNA	25.5 ± 4.9	172.85 ± 22.9	900.12 ± 65.23	3.61 ± 1.1	87.22 ± 16.9	10.32[g)]
pTPA-PA63 -LAMP1+ pUQ-PA63 Pro	90.8 ± 11.8	840.95 ± 276.9	1111.2 ± 34.8	41.6 ± 5.6	143.3 ± 20.8	5.66[g)]
pTPA-PA63-LAMP1+ pUQ-PA63 DNA	4.54 ± 0.98	110.0 ± 25.1	208.98 ± 22.5	39.2 ± 9.9	134.1 ± 18.5	7.56
rPA63	55.7 ± 10.7	135.17 ± 35.9	94.79 ± 31.9	17.8 ± 3.8	88.88 ± 22.9	1.07
rPA83	128 ± 21.1	360 ± 57.8	122.2 ± 34.8	17.3 ± 4.7	81.66 ± 33.9	1.50
PBS	1.26 ± 0.23	1.95 ± 0.11	1.79 ± 0.34	1.01 ± 0.23	2.66 ± 0.33	0.67
VECTOR	1.28 ± 0.56	1.12 ± 0.25	1.10 ± 0.56	1.23 ± 0.34	1.23 ± 0.45	0.89

[a)] Groups of 8–10 mice were immunized i.m. with different DNA constructs. Four weeks post the last immunization mice were euthanized to take out the spleens and spleen cells from the mice were cultured in the presence 10μg/ml of rPA63 protein to determine the cytokine concentration in the cell-free supernatant.

[b)] Cell-free supernatants were collected at 24 h, for IL-2, IL-4 and IL-12 measurement.

[c)] Cell-free supernatants were collected 72 h later (for IFN-γ and IL-10)

[d)] Indirectly measured as IFN-γ: IL-10 ratio.

[e)] Each value is a single mouse representative mean ±SE of 4-6 mice tested in a group in three independent experiments.

[f)] Significantly higher ($p<0.001$) ratio compared to all the groups bearing TPA, LAMP1 address tags as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[g)] Significantly higher ($p<0.01$) ratio compared to the pTPA-PA63 group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Table 3. Pre-challenge TNA titers in the serum of immunized mice.

	TNA titers [a], 10^3 ±SE	
Construct	**I[st] Boost**	**2[nd] Boost**
pTPA-PA63 Pro	0.325±0.08	0.450±0.10 [b), d)]
pTPA-PA63 DNA	0.230±0.07	0.390±0.09 [d)]
pUQ-PA63 Pro	≤0.05	0.110±0.02
pUQ-PA63 DNA	≤0.05	0.101±0.01
pTPA-PA63 -LAMP1 Pro	0.475±0.10	0.650±0.10 [b), d)]
pTPA-PA63 -LAMP1 DNA	0.410±0.09	0.625±0.19 [b), d)]
pPA63-LAMP1 Pro	0.290±0.07	0.400±0.15 [c)]
pPA63-LAMP1 DNA	0.250±0.10	0.350±0.10
pPA63-NATIVE Pro	≤0.05	≤100
pPA63-NATIVE DNA	≤0.05	≤100
pTPA-PA63 +UQ-PA63 Pro	0.215±0.08	0.365±0.09
pTPA-PA63 +UQ-PA63 DNA	0.200±0.09	0.305±0.08
pTPA-PA63 -LAMP1+ UQ-PA63 Pro	0.110±0.02	0.245±0.05
pTPA-PA63 -LAMP1+ UQ-PA63 DNA	0.100±0.04	0.205±0.09
rPA63	0.250±0.05	0.395±0.10 [c)]
rPA83	0.310±0.10	0.425±0.10 [c)]

[a)] Mice were immunized at days 0, 28 and 56; and TNA titers were measured in the serum of the immunized mice 14 days after each immunization. Titers after priming were ≤50 for all the groups. Corresponding values after I[st] and 2[nd] booster dose represent TNA titers obtained for a single mouse representative of 4-6 mice tested in a group in three independent experiments and results are expressed as mean ±SE.

[b)] Significantly higher (p<0.001) titers as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c)] Significantly higher (p<0.01) titers as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d)] Significantly higher (p<0.05) titers as compared to those groups that received their combinations with pUQ-PA63 chimera as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Table 4. Avidity indices of anti-PA antibodies in the serum of immunized mice.

Construct	Antibody avidity index [a]		
	Week 12	Week 14 (Challenge)	Week 16 (Post challenge)
pTPA-PA63 Pro	62	71	75 [b), d)]
pTPA-PA63 DNA	45	49	63 [d)]
pUQ-PA63 Pro	30	36	45
pUQ-PA63 DNA	25	34	40
pTPA-PA63 -LAMP1 Pro	80	85	92 [b), d)]
pTPA-PA63 -LAMP1 DNA	75	81	87 [b), d)]
pPA63-LAMP1 Pro	73	77	85 [c)]
pPA63-LAMP1 DNA	55	61	67
pPA63-NATIVE Pro	41	45	58
pPA63-NATIVE DNA	39	44	50
pTPA-PA63 +UQ-PA63 Pro	43	46	53
pTPA-PA63 +UQ-PA63 DNA	36	39	47
pTPA-PA63 -LAMP1+ UQ-PA63 Pro	57	67	72
pTPA-PA63 -LAMP1+ UQ-PA63 DNA	45	49	54
rPA63	75	78	82 [b)]
rPA83	80	86	75 [c)]
PBS	- [e)]	-	-
VECTOR	-	-	-

[a)] Avidity index= (endpoint titer in the presence of urea)/ (endpoint titer in the absence of urea)) x 100.

[b)] Significantly higher ($p<0.001$) avidity index as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c)] Significantly higher ($p<0.01$) avidity index as compared to the pPA63-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d)] Significantly higher ($p<0.01$) avidity index as compared as compared to those groups that received their combinations with pUQ-PA63 chimera as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[e)] No PA-specific antibody was detected from the serum of the control mice. These mice died after toxin challenge.

Table 5. Anti-PA and TNA titers post-challenge at different time points.

Group [a]	Total Anti-PA titers [b] ($10^3 \pm$ SD)					Letx Neutralizing titers [b] ($10^3 \pm$ SD)				
Weeks	10	12 [c]	14 [c]	16	18	10	12 [c]	14 [c]	16	18
pTPA-PA63 Pro	55.5±9.5	87.5±12.5	85±10.5	85±11.1	83±10.3	0.61±0.10	0.82±0.15	0.81±0.2	0.80±0.19	0.79±0.25
pTPA-PA63 DNA	47.0±8.0	78.5±9.2	75.9±10.2	75.5±7.2	72.0±8.0	0.46±0.15	0.70±0.2	0.69±0.1	0.69±0.2	0.67±0.15
pUQ-PA63 Pro	20.5±4.2	35.7±5.7	34.5±5.2	34±5.2	33.5±5.2	0.19±0.02	0.25±0.03	0.24±0.02	0.22±0.03	0.22±0.05
pUQ-PA63 DNA	19.6±4.2	32.6±4.2	32.5±4.2	31.0±2.5	30.0±6.6	0.17±0.02	0.23±0.01	0.22±0.03	0.20±0.02	0.19±0.02
pTPA-PA63-LAMP1 Pro	75.0±4.2	97.5±5.6	100±10.1	98.7±7.2	98.5±9.5	0.76±0.2	1.01±0.3	0.98±0.25	0.97±0.2	0.97±0.35
pTPA-PA63-LAMP1 DNA	69.5±10.5	95.7±10.2	99.5±12.7	97.7±13.5	97.0±9.7	0.69±0.1	1.00±0.1	0.99±0.2	0.99±0.3	0.97±0.2
pPA63-LAMP1 Pro	49.5±6.2	69.5±10.5	65.0±10.0	64.0±6.2	64.0±9.4	0.49±0.09	0.69±0.1	0.69±0.2	0.65±0.1	0.63±0.15
pPA63-LAMP1 DNA	41.0±8.2	57.8±7.6	57.5±4.1	56.0±5.2	55±8.5	0.37±0.1	0.55±0.1	0.55±0.14	0.54±0.1	0.54±0.07
pPA63-NATIVE Pro	10.5±2.1	20.5±3.2	20.5±4.5	19.5±2.2	19±5.7	0.15±0.02	0.19±0.03	0.20±0.06	0.19±0.02	0.15±0.05
pPA63-NATIVE DNA	10.5±3.9	18.8±2.0	19.0±3.0	19.6±5.0	18±5.0	0.15±0.09	0.18±0.02	0.22±0.05	0.20±0.01	0.19±0.04
pTPA-PA63 + UQ-PA63 Pro	25.0±5.5	38.6±7.5	38.5±4.3	35.5±4.2	35.0±5.2	0.29±0.05	0.41±0.09	0.40±0.1	0.39±0.15	0.39±0.1
pTPA-PA63 + UQ-PA63 DNA	22.6±5.2	35.8±6.3	35.5±5.5	34.0±4.2	32.0±4.7	0.25±0.02	0.39±0.05	0.39±0.09	0.37±0.05	0.37±0.9
pTPA-PA63-LAMP1+ UQ-PA63 Pro	30.5±9.2	41.5±9.5	40.5±8.8	40.0±9.5	39.0±9.0	0.39±0.05	0.50±0.1	0.50±0.15	0.49±0.15	0.49±0.13
pTPA-PA63–LAMP1+ UQ-PA63 DNA	27.6±4.2	33.8±5.2	32.5±6.5	32.8±4.4	31.9±2.4	0.31±0.08	0.45±0.12	0.45±0.15	0.41±0.28	0.41±0.14
rPA63	50.5±10.2	80±20.2	80.8±12.6	79.0±15.8	78.5±14.5	0.55±0.09	0.79±0.24	0.79±0.2	0.75±0.20	0.75±0.15
rPA83	53±15.2	88±10.0	87.5±13.7	87.6±15.2	85.5±18.6	0.58±0.1	0.81±0.2	0.80±0.15	0.79±0.21	0.79±0.2

[a] Control mice that received PBS and vector died after challenge.

[b] Mice (n=8-9 per group) were challenged at different time points post immunization and post-challenge total anti-PA and TNA titers were determined and represented as the reciprocals of dilutions of the serum of the surviving mice, ±SEM.

[c] Significantly higher ($p<0.001$) titers as compared those developed at week 10 as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

FIGURE.1.

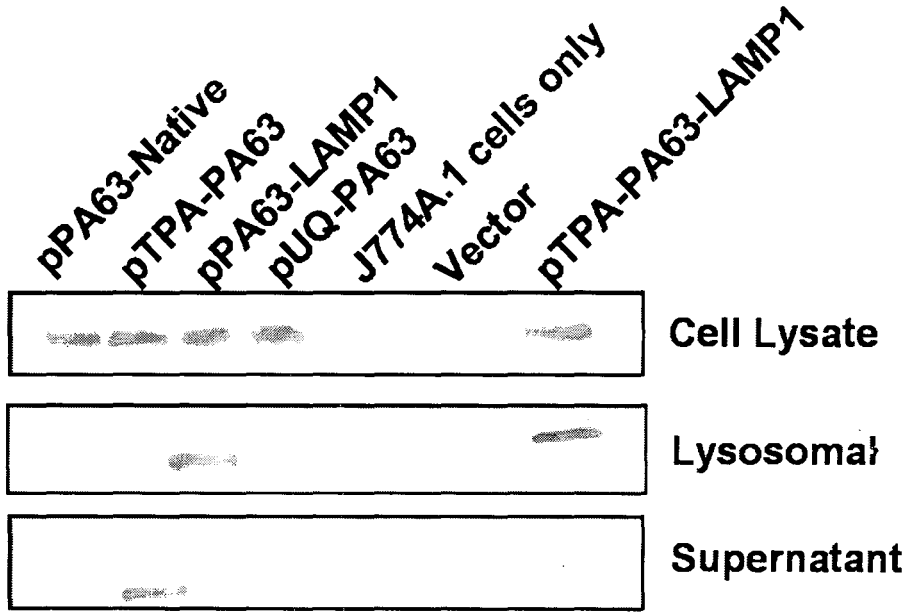

pPA63-Native
pTPA-PA63
pPA63-LAMP1
pUQ-PA63
J774A.1 cells only
Vector
pTPA-PA63-LAMP1
Cell Lysate
Lysosomal
Supernatant

FIGURE.2.

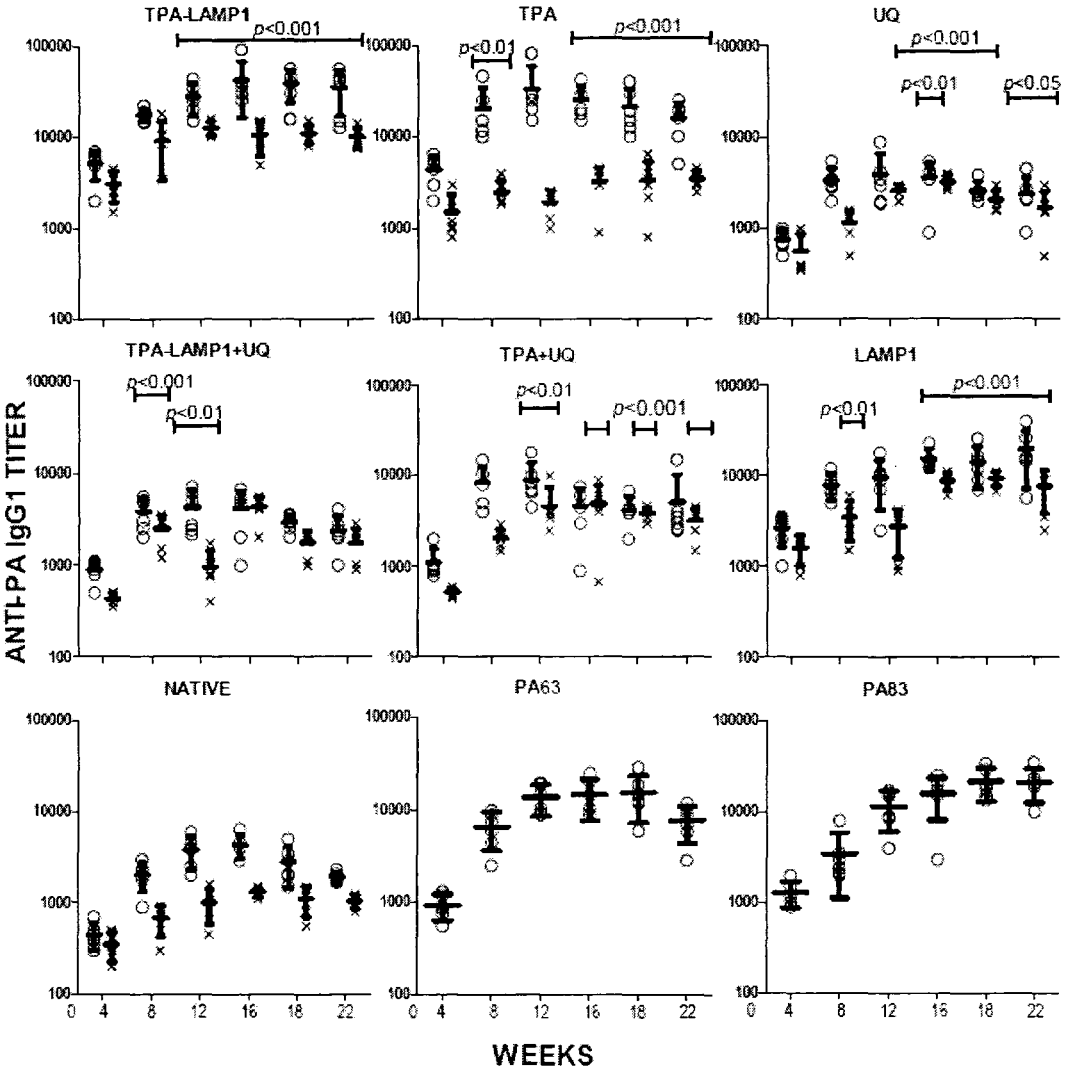

TPA-LAMP1
TPA
UQ
TPA-LAMP1+UQ
TPA+UQ
LAMP1
NATIVE
PA63
PA83
p<0.001
p<0.01
p<0.05
ANTI-PA IgG1 TITER
WEEKS

FIGURE.3.

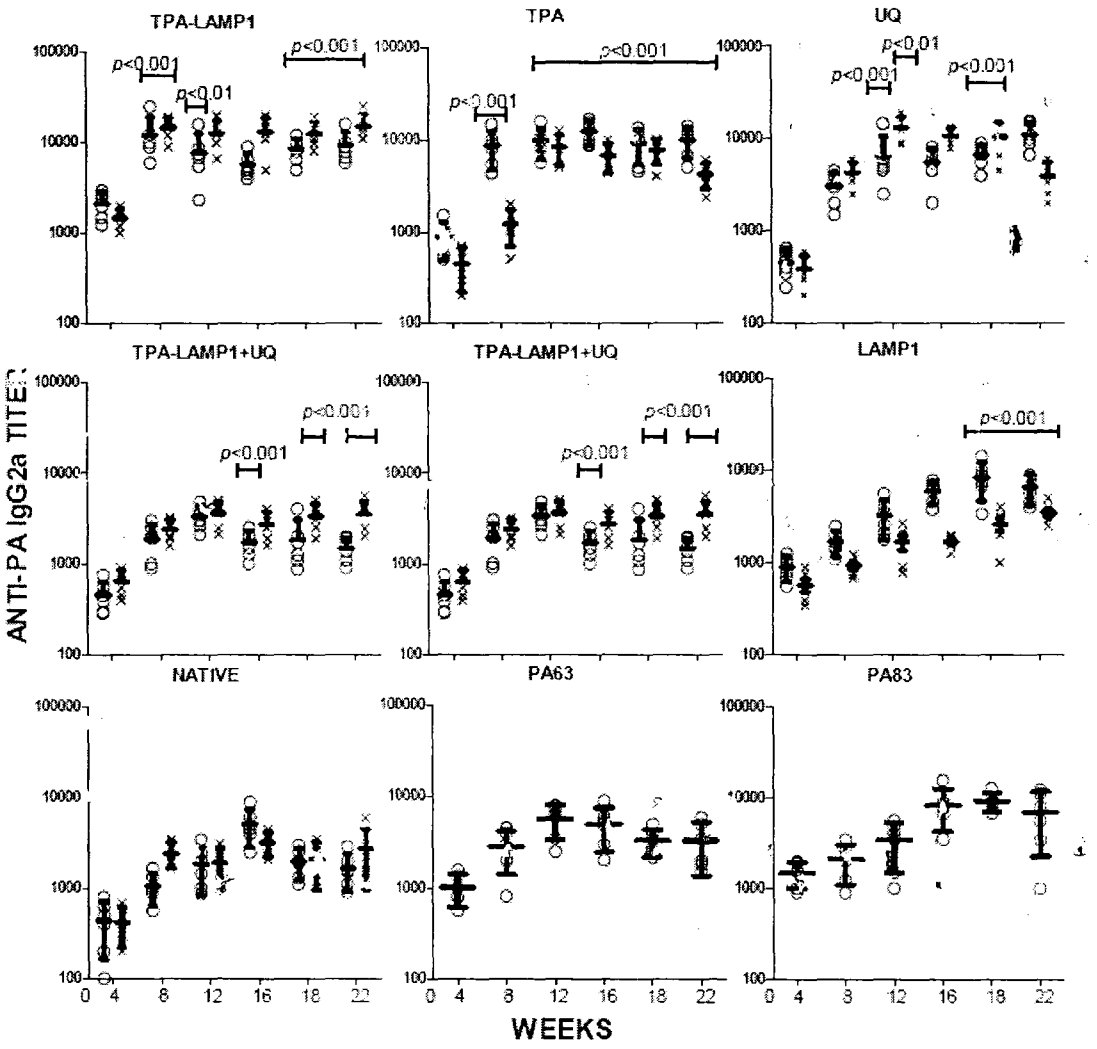

TPA-LAMP1
TPA
UQ
TPA-LAMP1+UQ
TPA-LAMP1+UQ
LAMP1
NATIVE
PA63
PA83
p<0.001
p<0.01
ANTI-PA IgG2a TITER
WEEKS
0 4 8 12 16 18 22
100 1000 10000 100000

FIGURE.4.

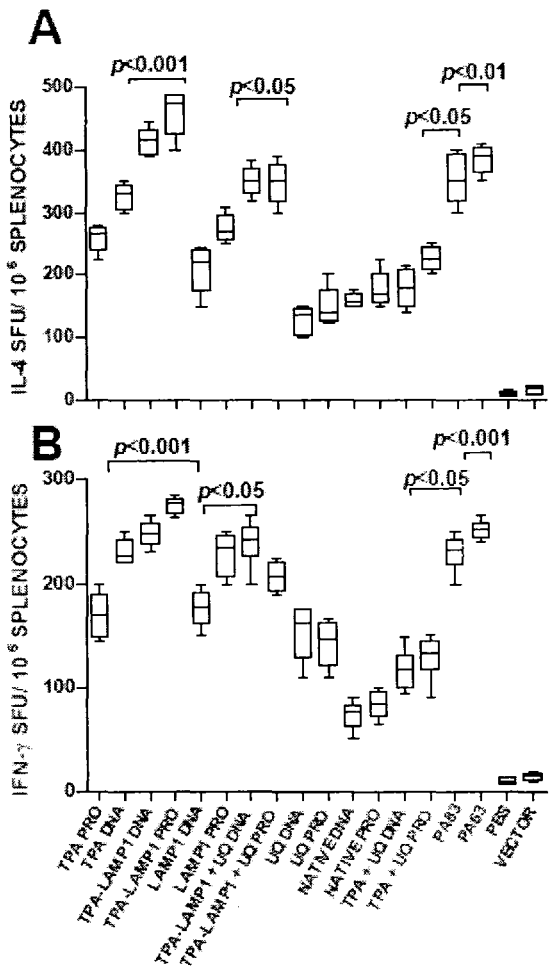

A
p<0.001
p<0.05
p<0.01
p<0.05
IL-4 SFU/ 10^6 SPLENOCYTES
500
400
300
200
100
0
B
p<0.001
p<0.05
p<0.001
p<0.05
IFN-γ SFU/ 10^6 SPLENOCYTES
300
200
100
0
TPA PRO
TPA DNA
TPA-LAMP1 DNA
TPA-LAMP1 PRO
LAMP1 DNA
LAMP1 PRO
TPA-LAMP1 + UQ DNA
TPA-LAMP1 + UQ PRO
UQ DNA
UQ PRO
NATIVE DNA
NATIVE PRO
TPA + UQ DNA
TPA + UQ PRO
PA63
PA63
PBS
VECTOR

FIGURE.5.

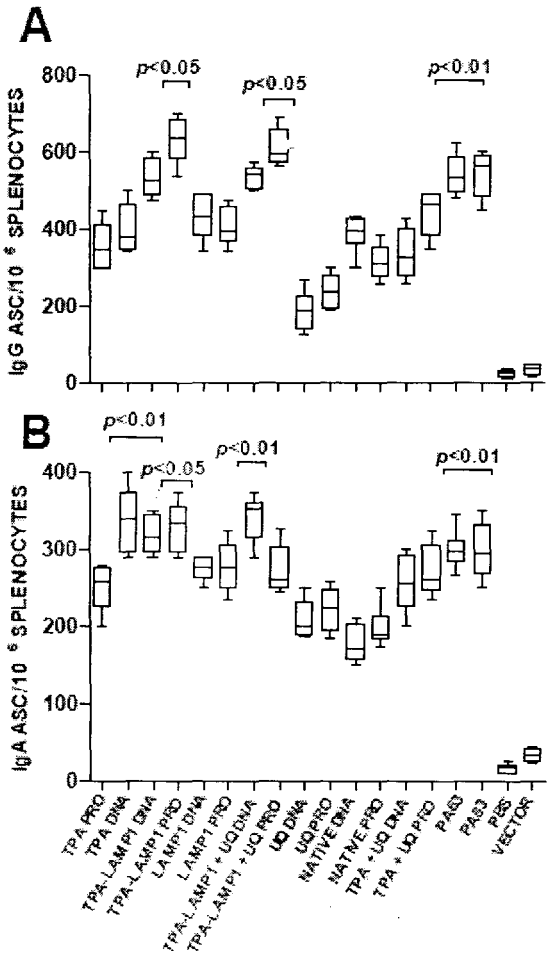


FIGURE.6.

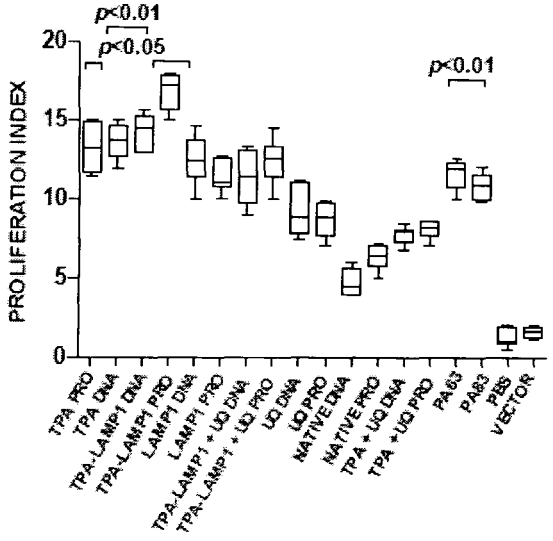
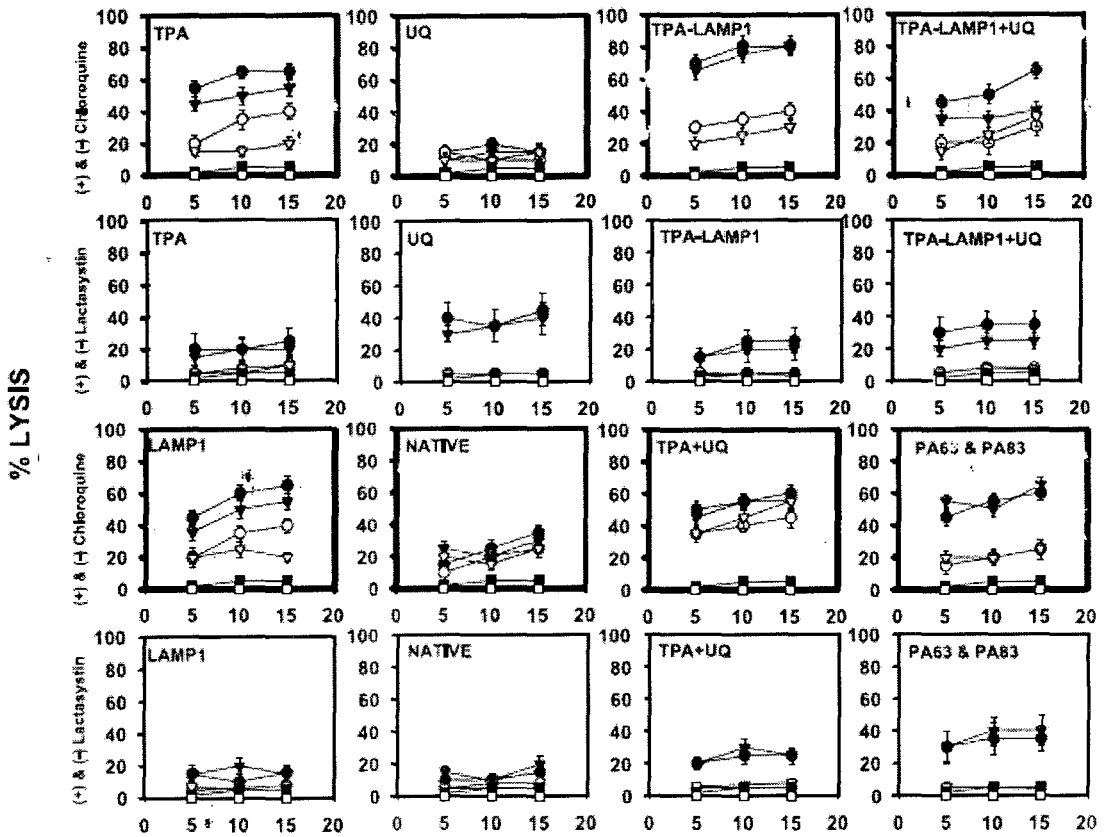


FIGURE.7.


% LYSIS
(+) & (-) Chloroquine
(+) & (-) Lactasystin
TPA
UQ
TPA-LAMP1
TPA-LAMP1+UQ
LAMP1
NATIVE
TPA+UQ
PA63 & PA83

SUPPLEMENTARY INFORMATION

Table 6. Primer sequences for cloning PA63 structural gene in DNA vaccine plasmids and pQE30 expression plasmid.

PRIMER SEQUENCE	N or C-TERMINAL LINKER	ADDRESS TAG
(A) Primers for cloning PA63 gene in DNA vaccine plasmids:		
Forward primer: (5'-GCGCAT **GCTCTTCCATG**CCTACGGTTCCAGACC G -3')	ATG (N-terminal)	All vectors
Reverse primer 1 (5'-GCAGAA**GCTCTTCGTTA**TCCTATCTCATAGCCTTTTTTA-3')	TTA (C-terminal)	Ubiquitin, Native, Secreted
Reverse primer 2 (5'- GCAGAA**GCTCTTCGGCC**TCCTATCTCATAGCCT TTTTTA G-3')	GCC (C-terminal)	Endosomal, Secreted-endosomal
(B) Primers for cloning PA63 gene in pQE30 expression plasmid:		
Forward Primer: (5'-CGC **GGATCC** GAT CCT ATC TCA TAG CCT TTT TTA G-3')	NIL	NIL
Reverse Primer: (5'-CGG **GGTACC** ATG CCT ACG GTT CCA GAC CG-3')	NIL	NIL

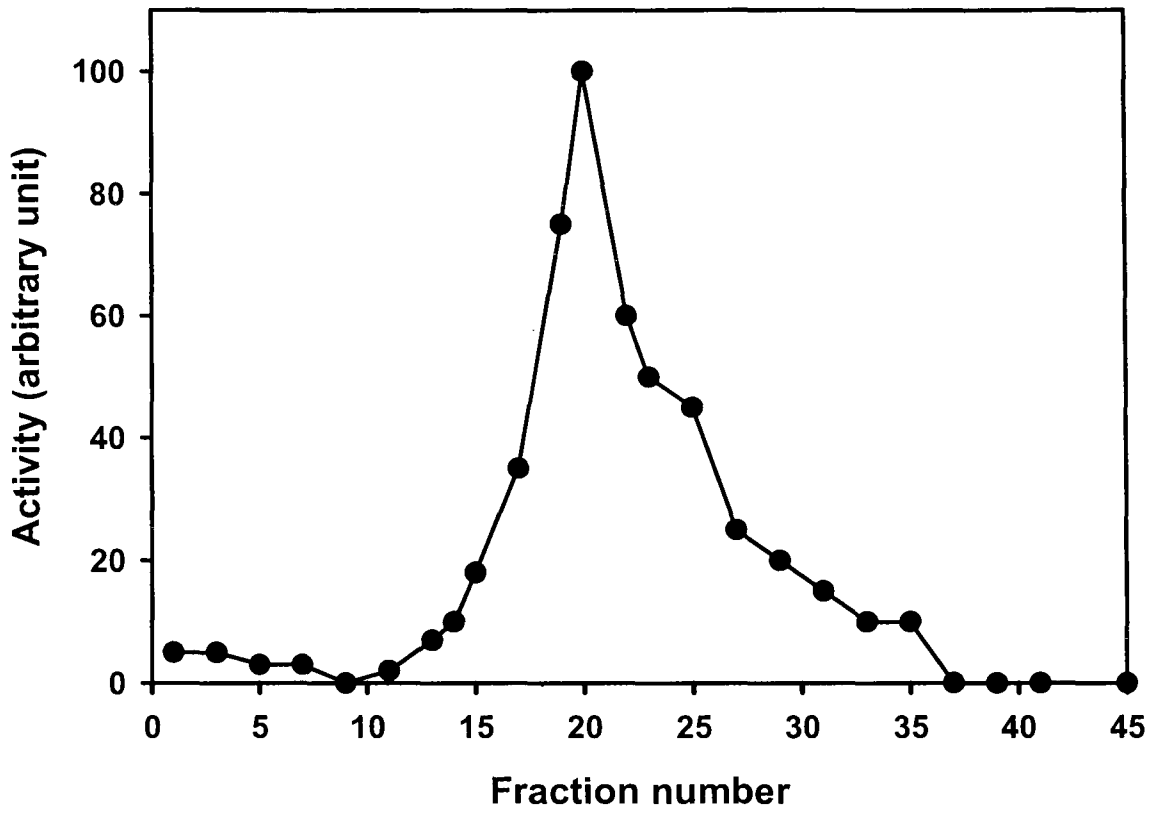


FIG. 9. Detection of β-hexosaminidase activity. β-hexosaminidase activity was measured in the various fractions obtained as a result of sub-cellular fractionation to separate and identify the Lysosomal fractions.

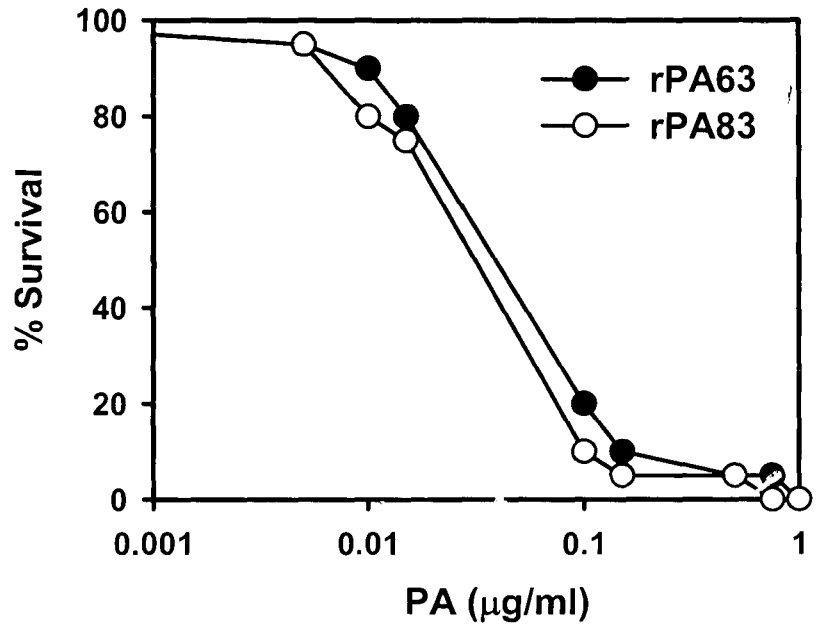


FIG.10.Biological activity of rPA63 as receptor binding moiety as compared to PA83. The biological activity of rPA63 as receptor binding moiety was established in a standard J774A.1-based cytotoxicity assay and compared with that of rPA83.

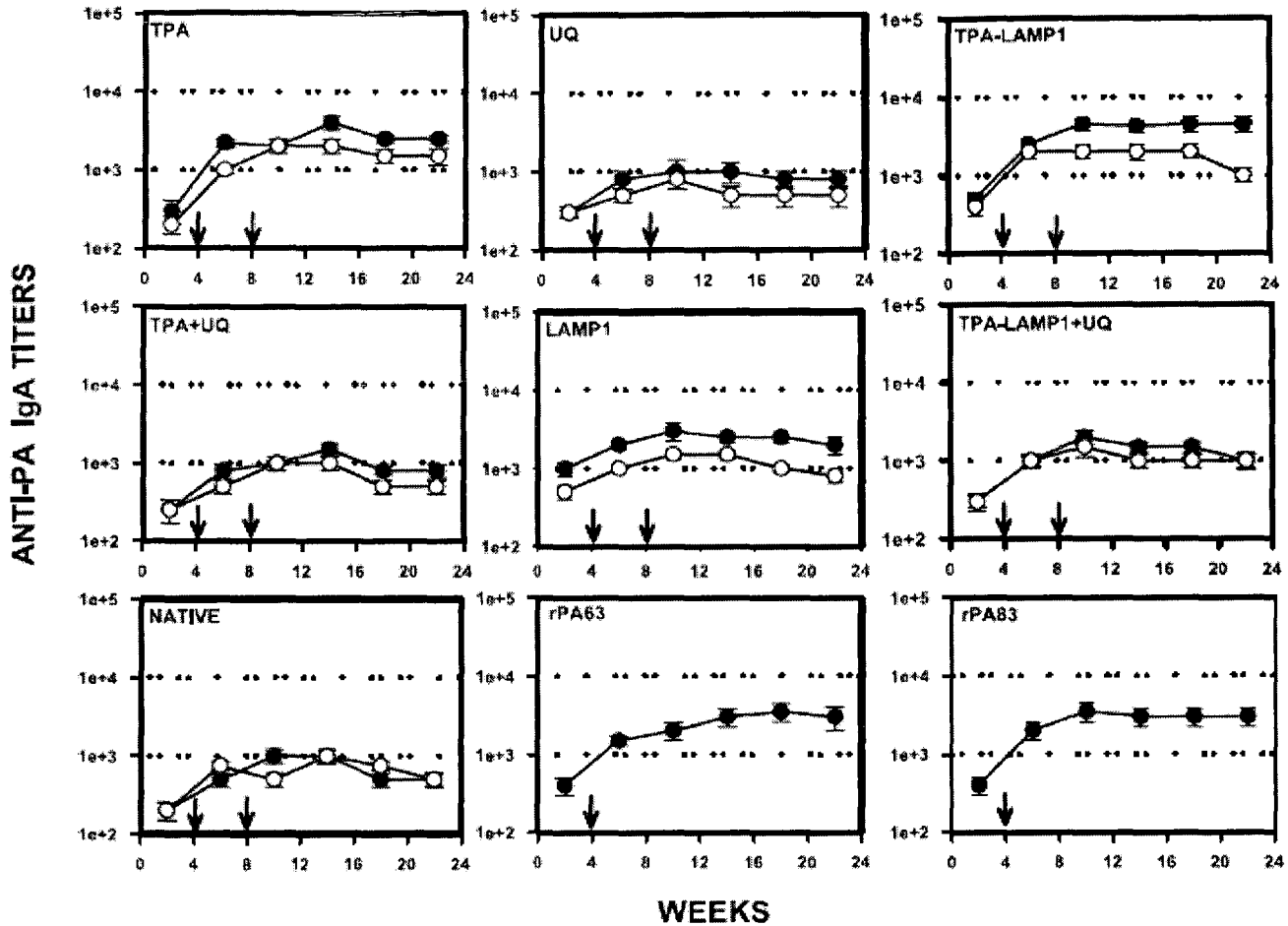


FIG.11.

FIG.11. Long-lasting IgA titers were generated in response to PA63-based DNA vaccine constructs in mice. *Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgA titers elicited following immunization were monitored till 22 weeks. Each curve represents IgA titers elicited by a single mouse representative of 6-7 mice tested in a group in two independent experiments and results are expressed as mean ±SE. Solid circles represent titers obtained for mice that received protein booster and open circles represent titers obtained for mice that received DNA booster. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgA titers.*

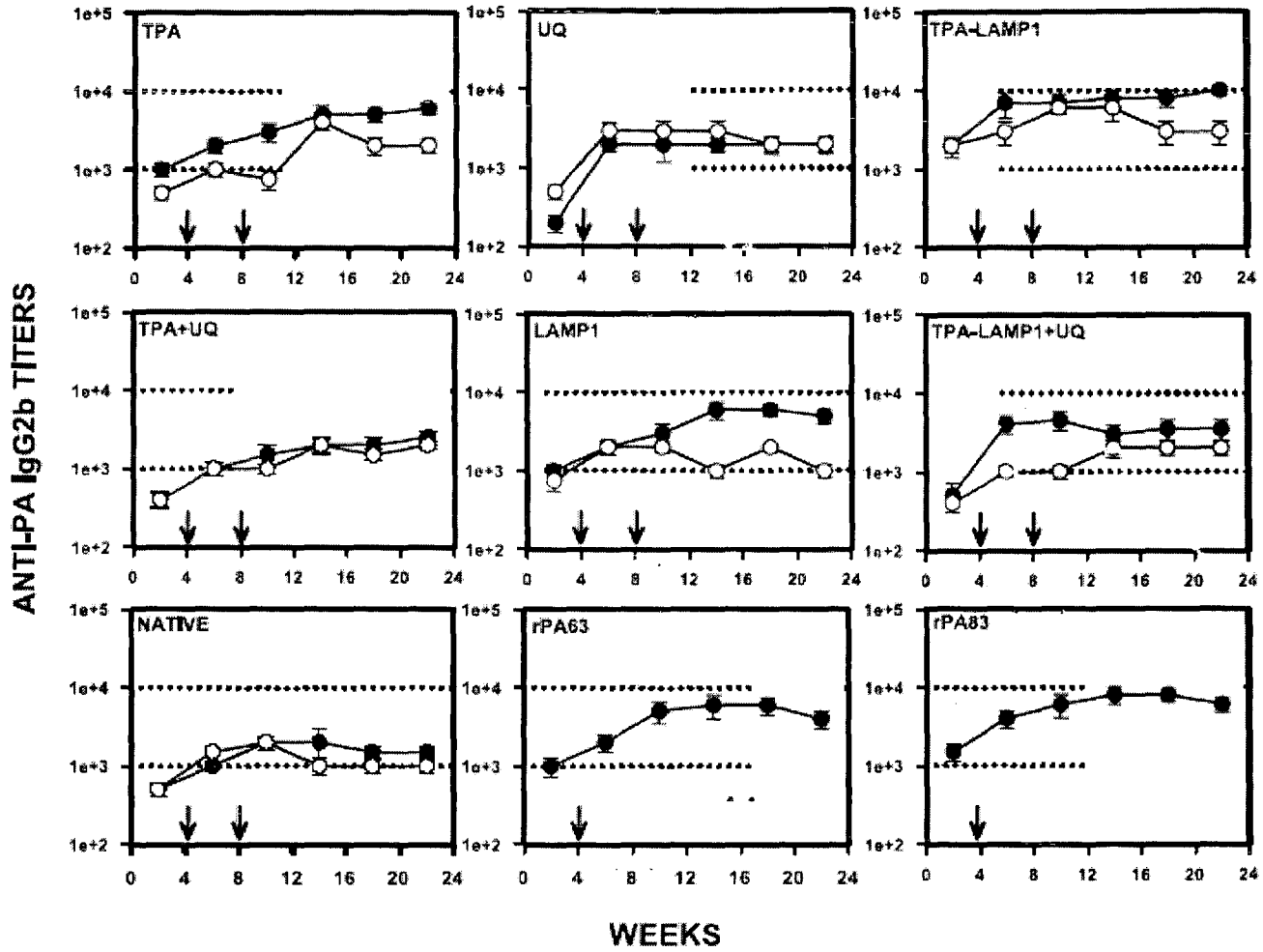


FIG.12.

FIG.12.High IgG2b titers were generated by DNA chimeras in mice. *Balb/c mice were immunized either thrice i.m. with DNA (100μg) or twice with DNA followed by s.c. boost with rPA63 protein (12.5μg) formulated with IFA on days 0, 28 and 56. Anti PA-IgG2b titers elicited following immunization were monitored till 22 weeks. Each curve represents IgG2b titers elicited by a single mouse representative of 6-7 mice tested in a group in two independent experiments and results are expressed as mean ±SE. Solid circles represent titers obtained for mice that received protein booster and open circles represent titers obtained for mice that received DNA booster. Control groups (PBS and vector-immunized) did not show any detectable anti-PA IgG2b titers.*

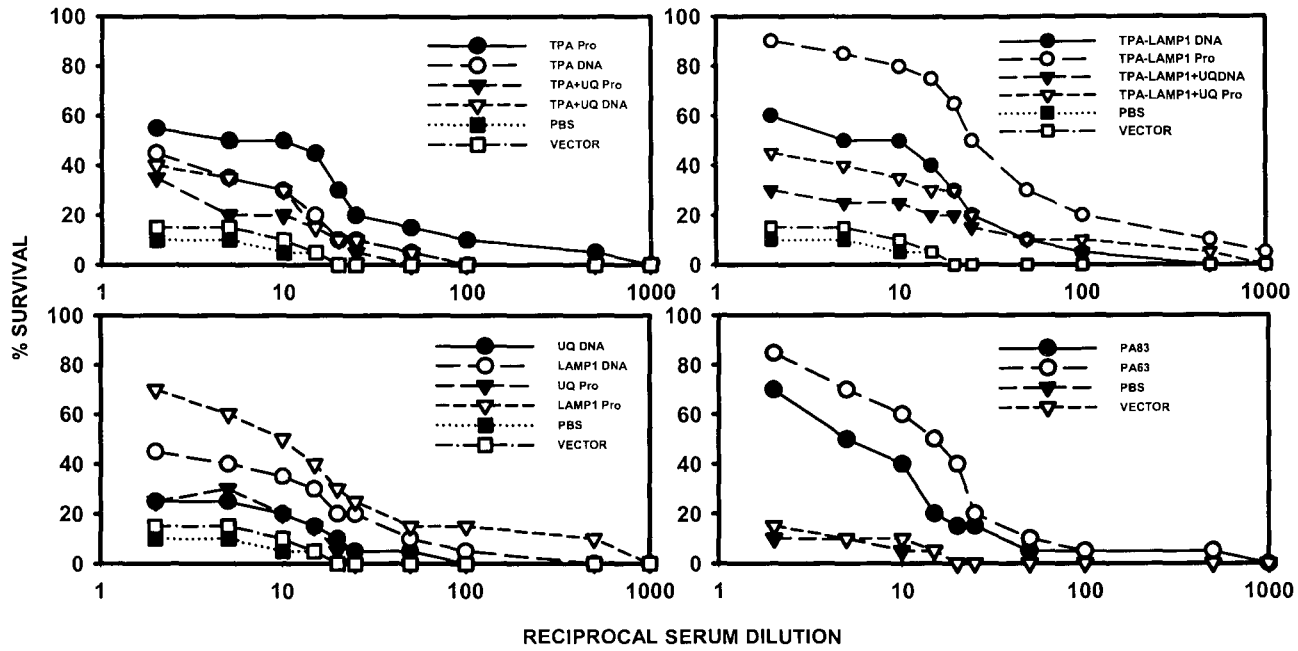


FIG.13. Efficient neutralization of lethal toxin by serum from immunized mice. *Various dilutions of the serum from DNA immunized mice were added together with Letx (1.0μg/ml each of rPA83 and rLF) to J774A.1 cells and incubated for 4h. Following that, MTT (Sigma-Aldrich) was added to a final concentration of 0.5mg/ml and the cells were incubated for a additional 30 min. Thereafter, the cells were lysed with acidic isopropanol (0.04-0.1N HCl in 90% isopropanol) and A450 of 100% viable cells was calculated from the average of four wells receiving no Letx. Results were expressed as the % survival of J774A.1 cells that survived the lethal shock.*

Paper Communicated

Genetic Immunization with GPI-anchored Anthrax Protective antigen raises combined CD1d- and MHC II-restricted Antibody Responses by Natural Killer T cell-mediated help

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ABSTRACT

Studies have paved way to the understanding that lipid rafts ultimately regulate the endocytosis of anthrax toxin via clathrin dependent pathway. Interestingly, GPI-anchored protein rich rafts have also been shown to be transported down to the endocytic pathway to reducing late endosomes. Taking advantage of this parallelism, we tried translating the anthrax toxin natural intoxication mechanism by administering a DNA chimera that encoded protective antigen attached to a mammalian GPI-anchor at its C-terminus (pGPI-PA). We also designed a chimera that had an additional N-terminal TPA leader (pTPA.GPI-PA) with an aim to target GPI-PA to ER where new CD1 molecules are synthesized. Analysis of antibody titers demonstrated successful priming and potential IgG titers after the first boost. In vitro cell proliferation studies revealed that there was a clonal expansion of $CD4^+NK1.1^+$ helper T-cell population which rapidly produced IL-4 in response to T cell receptor ligation. These cells provided direct B-cell help that aided IgG formation. Effector responses generated by NKT cells were found to be MHC II-independent and CD1d-restricted. In addition, the group pTPA.GPI-PA also displayed low magnitude MHC-II restricted (CD1d-independent) NKT cell and $CD4^+$ T cell helper responses in response to non-GPI form of PA which overall resulted in the heightened responses seen for this group. Importantly, DNA vaccination mediated the generation of high avidity neutralizing antibodies that mediated protection against lethal toxin challenge.

KEY WORDS: Anthrax; DNA vaccination; GPI-anchored protective antigen, Natural Killer T cell.

1. INTRODUCTION

Bacillus anthracis, the causative agent of anthrax, has developed an ingenious strategy to target cells by secreting a soluble proteinaceous exotoxin which belongs to a large family of binary (AB type) pore forming toxins. The brisance caused by this binary toxin sets in once the 'B' subunit (Protective antigen; PA83) binds cell surface anthrax toxin receptor (ATR), TEM8 and CMG2 [1, 2]. Further upon furin cleavage, B subunit (PA63) forms a pre-pore with a β-barrel structure, resulting from association of individual monomer loops [3, 4]. Pre-pore to pore conversion requires the endocytosis of this β-barrel structure which undergoes structural rearrangements triggered by the low pH in this compartment to form a membrane spanning pore [5, 6]. All this is undertaken to ensure the translocation of the catalytically active 'A' subunit (Edema factor; EF and Lethal factor; LF) into the cytosol where it can hijack the cellular machinery [7]. Interaction with EF and LF with PA63 occurs at the cell surface after the heptamerization has occurred [8]. Once in the cytosol, EF, an adenylate cyclase, upon calmodulin binding undergoes structural rearrangements that leads to its activation and induces substantial increase in conversion of intracellular ATP to cAMP [9]. Subsequently, water homeostasis and cellular signaling of host are disrupted, leading to edema during cutaneous anthrax infection [9, 10]. LF, on the other hand, a zinc-dependent metalloprotease, cleaves short N-terminal fragments from mitogen or extracellular signal-regulated protein kinase kinasel (MEK1), MEK2 and MEK3, the upstream activators of

ERK1, ERK2 and p38, respectively [11- 13] that disrupts antigen receptor signaling resulting in macrophage function inhibition and cell death [13, 14].

The depredation thus, resulting from anthrax toxin action in affected individuals has riveted attention. Early treatment is essential, as the disease shows a rampant aggression at which antibiotics are no longer effective owing to the accumulation of a lethal level of toxin, even though the organism is sensitive to the agent. Post exposure prophylaxis is a combination of an antibiotic (ciprofloxacin) and a licensed human vaccine AVA (Anthrax Vaccine Absorbed). But AVA suffers from the chief criticism of incomplete characterization of the actual composition of the vaccine preparation which often results in reactogenicity and generates the need for frequent administration of boosters [15]. Therefore, the current scenario necessitates retrospection and search for a better and; safer anthrax vaccine.

In this context the potential of a novel approach that uses DNA for vaccination has also been exploited. Most of the DNA vaccination strategies, tested so far against anthrax, have mainly relied on DNA-adjuvant combinations [16-28]. Few others have aimed at abrogating initial events of anthrax toxin action such as binding of PA to ATR, with an aim to counteract the disease before its inception [29]. Studies have shown that ATR resides in the glycerolipidic i.e. non-raft regions of the plasma membrane. However, upon binding and heptamerization of PA, the toxin-receptor complex associates with lipid-raft like domains and undergoes rapid endocytosis via clathrin-dependent pathway [30]. This process subsequently leads to the translocation of the enzymatic subunits into the cytosol through the protective antigen pore. This kind of behavior parallels B-cell receptor which also undergoes ligand-dependent clustering and raft association [31], and is subsequently internalized via clathrin dependent mechanism [32].

The observation that ATR couples raft translocation and mediates endocytosis of EF/LF along with the oligomerization of PA made us hypothesize that a DNA chimera encoding protease-cleaved fragment of PA (PA63) attached to a C-terminal glycosylphosphatidyl inositol (GPI) anchor sequence might ensure lateral association of the cell-surface GPI-anchored PA with liquid-ordered, cholesterol- and sphingolipid-rich domains or rafts. Biochemical, morphological and functional approaches have been able to trace that GPI-anchored protein rich rafts are transported down to the endocytic pathway to reducing late endosomes in mammalian cells [33]. Apart from that, GPI-anchoring has been attributed to be a positive signal for internalization into rab5-independent (rab5, a small GTPase located in early endosomes) tubular-vesicular endosomes also responsible for fluid-phase uptake [34]. Therefore, adoption of such an approach opens up a possibility that GPI anchored PA will undergo its natural cycle upon endocytosis that might improve DNA vaccine potency. As a matter of fact, it is also well documented that CD1 molecules (MHC-I like glycoproteins) survey the endocytic pathway to intersect and bind lipid antigens [35]. Both human and muroid CD1d molecules (Group 2 CD1 molecules) have been shown to bind to GPI-anchored proteins and present them to a variety of NKT cells that function against infection with pathogens [36]. Group 2 CD1 (CD1d) molecules have also been shown to control humoral immunity against parasites [37], elicit cell-mediated immune responses against tumors [38], and induce secretion of large amounts of both inflammatory IFN-γ and immunoregulatory interleukin (IL)-4 and IL-10 cytokines [39, 40]. Thus, GPI-anchoring of PA might as well aid its presentation in context with CD1 molecules that have the advantage of limited allelic polymorphism yet specialized to bind and present a large repertoire of lipids and glycolipids to T cells.

Based on this rationale we constructed DNA chimera encoding GPI-anchored form of PA utilizing the mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. The GPI signal appears to have been conserved, however, a mammalian GPI signal was specifically attached keeping in mind earlier studies that demonstrate that GPI signals from parasitic protozoa are not recognized by mammalian cells [41]. We also designed a chimera in which PA was attached to an N-terminal TPA leader alongwith a C-terminal GPI anchor. TPA is expressed by vascular smooth muscle and binds to a specific cellular receptor

p63, which occurs in fibroblasts as an intracellular protein associated with the ER [42, 43]. The TPA leader peptide can therefore, target the expressed antigen directly to the ER thus, obviating the need for the antigen to be processed and translocated to this structure [44]. So, we wanted to find out what impact, if any, it would have on DNA vaccine potency as ER is also the site where new CD1d molecules (the only muroid CD1 molecule) are synthesized.

Therefore, with these observations in mind we designed the present study and we successfully illustrated the generation of not only classical MHC II-restricted immunoglobulin (Ig) responses but also non-classical CD1d-restricted Ig responses. Importantly, DNA vaccination mediated the generation of high avidity toxin neutralizing antibodies which efficiently protected animals against anthrax lethal toxin challenge.

2. MATERIALS AND METHODS:

2.1. DNA vaccine plasmids and recombinant proteins. DNA manipulations were performed according to standard molecular biology procedures [45] using the *E. coli* DH5α strain. Protease-cleaved fragment of the structural gene for protective antigen (PA63) was amplified by PCR from *Bacillus anthracis* (Sterne strain) pXO1 plasmid using the following primers: Forward primer: (5'-GCG CAT GCT CTT CCA TG C CTA CGG TTC CAG ACC G -3'), and Reverse primer 2 with GCC alanine linker (5'-GCA GAA GCT CTT CGG CCT CCT ATC TCA TAG CCT TTT TTA G-3'). The primers were designed to generate 5'-ATG and 3'-TAA for address tags that correspond precisely to start and stop codons (for N-terminal, TPA Leader bearing plasmid) or 5'-ATG and 3'-GGC alanine linker for address tags that facilitate C-terminal extensions needed for trafficking (GPI anchor). The amplified product was cloned in a eukaryotic expression vector bearing the targeting signals. Recombinant PA (rPA) was obtained from chimera pMWpag cloned in our lab previously [46]. rPA was expressed as a fusion protein with 6x histidine tag in the *E.coli* M15 strain and was purified on Ni^{2+}-NTA column to more than 95% homogeneity from inclusion bodies as described previously [46]. Peptides (mapped epitopes of PA) used for in vitro stimulation for cells were synthesized commercially (Clover Scientific Pvt. Ltd., India) at >95% purity. Peptide sequences are described in the Table1. In addition peptides representing the epitopes of PA attached to a mammalian GPI-anchor sequence (mentioned in Table 1) were also included to determine whether the GPI anchored PA specifically accounted for any of the seen differences in immunological behavior of the proteins. The resulting PA peptide sequences with an additional GPI anchor sequence were also synthesized commercially at > 95% purity.

2. Plasmid DNA preparation. Plasmid DNA was prepared from overnight cultures of transformed DH5α bacteria in Luria Bertani Broth (Amersham) plus 50 μg/ml kanamycin sulfate (Amersham) and processed by using Endo-free Giga kits (Qiagen, Valencia, CA).

2.3. Transfection and Immunoblotting. J774A.1 mouse MΦ-like cells were seeded at a concentration of 2-3 × 10^7 cells into a 75-cm^2 flask (Corning costar) until the cells reached approximately 50–70% confluence. Plasmid DNA transfection was performed with LipofectAMINE 2000 (Invitrogen) reagent, as specified by the manufacturer. For Western blot analysis, the transfected cells were washed twice with PBS (pH 7.4) and lysed in lysis buffer (20 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate) 48 h post-transfection to prepare total cell lysates. Cell membrane protein fraction was prepared by Qproteome membrane protein kit (Qiagen) according to the manufacturer's protocol. The presence of cell membrane in the fractions was determined by the associated NADH oxidase activity [47]. Culture supernatant proteins were precipitated by ice-cold acetone. The proteins from the total cell-lysates, cell membrane fractions and culture supernatants were subjected to SDS–polyacrylamide gel electrophoresis under denaturing conditions with 50 mM dithiothreitol (DTT). Proteins from the gel were transferred on to a nitrocellulose membrane and probed with rabbit anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG (Amersham biosciences). Blot was developed using BCIP/NBT (Sigma-Aldrich) as substrate.

2.4. Vaccination and lethal toxin challenge. Six- to eight-week-old female swiss albino outbred mice (National Institute of Nutrition, Hyderabad, India) were immunized intramuscularly (i.m.) with 100 μg of DNA suspended in phosphate-buffered saline (PBS; 50 μl per hind leg) administered via a 26-gauge, 1-ml hypodermic needle. Following that all the mice received two DNA boosters (100μg each, i.m.) on day 28 and 56. Sera was obtained from blood samples collected from the retro-orbital plexus bleeding prior and post immunization. On different time intervals (12, 14, 16, 18, 20 weeks) post immunization, DNA vaccinated mice were challenged with anthrax lethal toxin (Letx, PA83=50μg and LF=22μg, ≈ 4-5 LD_{50}) injected intravenously via tail vein, and the challenged mice were closely monitored for 21 days. All experiments were performed in accordance with 'Indian Animal Ethics Committee' regulations.

2.5. ELISA detection of anti-PA reactivity in mouse serum. The anti-PA reactivity of immunized animal sera was determined by direct ELISA. Briefly, microtiter plates were coated with rPA (10μg/ml) in PBS. Following blocking and washes in PBS-Tween 20 (0.2%), plates were incubated with goat anti-mouse IgG and IgM HRP conjugate (Santacruz Biotechnology) for 1 h at 37°C. After washes in PBS-Tween 20 (0.2%), plates were developed using TMB substrate (Amersham biosciences). The reaction was stopped with 1N sulfuric acid, and the plates were analyzed at 450 nm in an ELISA reader (Benchmark Plus Microplate spectrophotometer, BioRAD). The negative controls included sera from mice immunized with PBS and vector. Endpoint antibody titers were defined as the last reciprocal serial serum dilution at which the absorption at 450 nm was greater than two times the background signal detected.

2.6. Lethal toxin neutralization assay. The protective effects of PA-specific Abs were determined using a previously described assay [48] that measures their capacity to protect the J774A.1 MΦcell line from Letx. Briefly, J774A.1 MΦ ($5x10^4$ MΦ/well) were seeded in 96-well, flat bottom plates. After 12 hrs of incubation, serum dilutions were added together with Letx (187.5ng/ml each of PA and LF, ~ 4-8 fold toxin dose) [48] and incubated for 4h. Viable MΦ were enumerated after addition of MTT (Sigma-Aldrich). The A_{450} of 100% viable cells was calculated from the average of four wells receiving no Letx. The average of duplicate samples was used to calculate titers, defined as the reciprocal of the highest dilution of serum that gives an $A_{450} \geq 90\%$ of the value of wells receiving no Letx.

2.7. In vitro cell proliferation assay. After isolation, 4 x 10^7 spleen lymphocytes from naive or DNA vaccinated mice were cultured in the presence of pooled peptides [either GPI-attached PA peptides or non-GPI PA peptides (10μg/ml of each peptide)] with IL-2 (10 U/ml) for a period of 4 days. After that $NK1.1^+TCR\text{-}\alpha/\beta^+$ cells and $CD4^+$ T cells were analyzed by flow cytometry using FACS Vantage™ instrument (Becton Dickinson). All mAbs used in these experiments (mAbs against NK1.1, CD4, and TCR-α/β) were purchased from BD PharMingen. The details of the staining and sorting have been described previously [49]. Clonal expansion in the presence of GPI-anchor peptide only (Table.1) was subtracted to negate any proliferation response generated specifically against the glycerolipidic region.

2.8. Isolation of lymphoid cell subsets by FACS. Spleen cells were incubated on nylon wool column for 45 min, and the nonadherent cells were used for the isolation of NKT cells and $CD4^+$ T cells by cell sorting using a FACS Vantage™ instrument (Becton Dickinson). All mAbs used in these experiments (mAbs against NK1.1, CD4, and TCR-α/β) were purchased from BD PharMingen. Unless noted otherwise, $CD4^+NK1.1^+TCR\text{-}\alpha/\beta^+$ cells were used as purified NKT cells. The stained cells were isolated using the FACS Vantage™. The purity of the sorted cells was >98%.

2.9. In vitro IL-4 production. NKT cells and $CD4^+$ T cells (10^5 cells/ml) were incubated with pooled peptides [either GPI-attached peptides or non-GPI peptides (10μg/ml of each peptide)] in the presence of splenocyte APCs (syngeneic splenocytes that were pulsed with peptides and then given mitomycin-C treatment). Antigen

induced IL-4 production was determined in the presence and absence of anti-MHC II, anti-MHC I and anti-CD1 monoclonal antibody (Pharmingen, CA , USA). Cell-free culture supernatants were harvested 24 hrs post stimulation and cytokine-specific sandwich ELISA was performed using OptEIA kit for the specific cytokine (BD Pharmingen) according to manufacturer's protocols. IL-4 production in the presence of GPI-anchor peptide only (Table.1) was subtracted to negate non-specific stimulation due to the glycerolipidic region only.

2.10. B-lymphocyte purification. After isolation, splenocytes were incubated with anti-CD43 and anti-Mac-1 antibody-conjugated microbeads (Miltenyi Biotec). The bead-bound cells (positive fraction) were separated from unbound cells (negative fraction) using an AutoMacs magnetic cell sorter. The enriched cell population was subjected to multiparameter fluorescence-activated cell sorting (FACS) analysis to sort expressing B220, a marker present on cells committed to the B lineage [50]. The preparation was 96% $B220^+$, the few contaminants were $CD43^+/Mac\text{-}1^+$.

2.11. Helper Assays. B lymphocytes (2.5 x 10^5/ml) were co-cultured in the presence of NKT cells, $CD4^+$ helper T cells or alone in the presence of either, GPI or non-GPI-PA peptides (pooled peptides, 10μg/ml) and IL-2 (10U/ml). Antigen specific IgG production was quantified upon addition of anti-class II and anti-CD1 mAb by ELISPOT. IgG production in the presence of GPI-anchor peptide only (Table.1) was subtracted to negate non-specific stimulation due to the glycerolipidic region only. Spots were developed using Alkaline Phosphatase-linked secondary antibody and BCIP/NBT (Sigma-Aldrich) as substrate. Results were expressed as number of SFU (Spot Forming Units) per 10^6 cells.

2.12. Determination of avidity of PA-specific IgG antibodies. Antibody avidity was measured by ELISA using urea as chaotropic agent to dissociate low-affinity antigen-antibody complexes [51]. Briefly, serum samples (in twofold dilutions) were incubated for 1 h at 37°C in ELISA plates coated with rPA. Plates were washed with PBST and soaked with 4 M urea for 10 min. After subsequent washes with PBST, bound IgG was detected with HRP-linked anti-mouse IgG by TMB substrate. Endpoint titers for each serum sample were determined, in the presence and absence of urea. Results were expressed as avidity index, calculated as the percentage of residual activity (endpoint titer) using the following formula: (endpoint titer in the presence of urea)/(endpoint titer in the absence of urea)) x 100. Positive and negative samples of known antibody titer (calibrated controls) were run in each assay. During the standardization of the assay, several concentrations of urea (0 to 8 M) were tested, and 4 M was selected as optimal; higher urea concentrations completely abrogated antibody binding.

2.13. Statistical analysis. The experimental data were analyzed by software programs Sigma Plot 8.1 or Excel (Microsoft) and were expressed as mean ± SE. The statistical significance of differences was analyzed by a two-tailed Student's t-test for independent groups (followed by Bonferroni's correction to adjust for multiple comparisons). A P-value of <0.05 determined was considered statistically significant. Correlation coefficients were determined by linear regression analysis.

3. RESULTS:

3.1. Transfection of DNA vaccine chimeras in J774A.1 Φ-like cells followed by detection with western blotting. The structural gene for PA63 was cloned in DNA vaccine plasmids bearing the address tags. Authenticity of the DNA constructs was evaluated by transient transfection in J774A.1 mouse MΦ-like cells followed by subcellular fractionation and immunoblot analysis. (Fig.1). Results indicated that the PA protein along with the GPI and TPA signal was being properly recognized by the mammalian cells. The DNA chimeras, pGPI-PA and pTPA.GPI-PA, expressed membrane-anchored form of PA (Fig. 1). Along with that the chimera pTPA.GPI-PA also mediated secretion of PA in the culture supernatants.

3.2. Significant serum antibody responses were generated in mice. A correlation between the presence of anti-PA antibody in serum and

protection against lethal challenge has been shown previously [52, 53]. Therefore, the delivery of DNA vaccine chimeras and generation of measurable humoral responses to PA in the serum of the immunized mice was evaluated by direct ELISA. All pre-immune serum titers of IgG and IgM antibodies against PA were negative. Following i.m. administration, 100% of the mice had strong serum IgM titers (Table. 2) which peaked after priming and then declined after administering the first and second booster dose. Pronounced PA-specific IgG titers were demonstrated by groups that were immunized with chimeras encoding the GPI-anchored form of PA. Highest titers were elicited by the chimera pTPA.GPI-PA (≈100,000), followed closely by the group pGPI-PA (≈79,000) after the second boost. Importantly, these titers were maintained until week 20 post priming (Table 3). The group immunized with pPA63-native also mounted significant IgG titers as compared to the control mice but the relative titers were lower. Titers after the initial priming were ≥ 3000 and after the first and second booster, titers increased to ≈10,000 and ≈20,000 respectively (Table. 2). Evaluation of Letx neutralization titers revealed that Letx neutralization activity appeared in the serum of animals only after the first booster dose (Table. 2). Neutralization titers were approximately 2-3 times higher in the serum of animals immunized with pTPA.GPI-PA and pGPI-PA than the animals vaccinated with the native construct lacking the GPI anchor. Peak lethal toxin neutralization titers developed around 14 weeks post the last immunization (Table. 3). Sera from control mice did not neutralize anthrax lethal toxin at all.

3.3. $CD4^+$ $NK1.1^+$ helper-T cells were generated upon vaccination with GPI anchored forms of protective antigen. Conventionally, IgG responses require MHC-II restricted recognition of the peptide fragments by $CD4^+$ helper T cells. However, IgG responses to GPI-anchored protein antigens has been found to be regulated in part through CD1d-restricted recognition of the GPI-moiety by $CD4^+$ $NK1.1^+$ helper T cells [36]. Therefore, we tried to decipher which one of the effector T-cell subset was responsible for B-cell help and hence the generation of immunoglobulin (Ig) responses in vaccinated mice. Flow cytometric analysis revealed that there was a marked increase in both the absolute and relative numbers of $CD4^+$ $NK1.1^+$ helper T-cell population after 5-day culturing in the presence of GPI-attached PA peptides (Table 4). The relative numbers of $CD4^+$ $NK1.1^+$ helper T cells after in vitro proliferation was astonishingly higher in mice immunized with chimera pGPI-PA (75.7%) and pTPA.GPI-PA (71.5%), as compared to those immunized with pPA-native (15%). Importantly, there was also proliferation of $CD4^+$ α/β TCR^+ cells, in vitro, for the group pTPA.GPI-PA. Though this population corresponded only to 18.7%, it proliferated rapidly when it was presented with non-GPI PA peptides giving rise to appreciable number (45.3%). The NKT cell population from this group also proliferated rapidly in the presence of non-GPI PA peptides but the relative number of NKT cells proliferating in response to GPI-attached PA peptides (71.5 %) was quite higher as compared to those proliferating in response to non-GPI PA peptides (39.7 %). The group immunized with the chimera pPA-Native on the other hand solely gave rise to $CD4^+$ α/β TCR^+ cells in the presence of non-GPI PA peptides only. Overall, chimera pTPA.GPI-PA resulted in proliferation of both $CD4^+$ α/β TCR^+ and $CD4^+$ $NK1.1^+$ helper T-cell population whereas pGPI-PA chimera resulted in clonal expansion of $CD4^+$ $NK1.1^+$ helper T-cell population in majority, in response to GPI-attached PA peptides. Also, the NKT cell population from the group pTPA.GPI-PA responded well against both the forms of PA whereas the one from pGPI-PA responded efficiently only against the GPI-anchored form.

3.4. Combined CD1d- and MHC II-restricted IL-4 responses were generated by chimera pTPA.GPI-PA. Fig.2. shows IL-4 production by $CD4^+$ $NK1.1^+$ helper T cells isolated from immunized mice when co-cultured with splenocyte APCs. NKT cell-mediated IL-4 production in the presence of GPI-attached PA peptides was completely abrogated by anti-CD1 mAb in both the groups that received pTPA.GPI-PA and pGPI-PA (Panel A & E). These responses on the other hand were completely unaffected by anti-MHC II mAb pointing that GPI-anchored PA was efficiently processed and presented in context with CD1 molecule to NKT cells in these groups. The group immunized with pPA-native did not

show any NKT cell mediated IL-4 production in the presence of GPI-attached PA peptides (Panel I). NKT cells isolated from the group pTPA.GPI-PA also showed NKT cell mediated IL-4 production in the presence of non-GPI PA peptides (Panel F). Interestingly, these responses were totally abrogated by anti-MHC II mAb but were completely unaffected by anti-CD1 mAb. Also, evaluation of $CD4^+$ α/β TCR^+ T cell-mediated IL-4 responses indicated that the $CD4^+$ T cells isolated from the group pTPA.GPI-PA and pPA-Native produced IL-4 only when they were presented with non-GPI PA peptides (Panel H & L). This lysis was completely abrogated by anti-MHC II mAb and was totally unaffected by anti-CD1 mAb indicating that the antigen was presented in context with MHC-II molecules. On the other hand IL-4 production by $CD4^+$ helper T cells isolated from the group pGPI-PA was insignificant in the presence of both the forms of PA (Panel C & D). Overall, the results provided evidence that $CD4^+NK1.1^+$ helper T cells from the group pTPA.GPI-PA mediated CD1d- and MHC-II restricted IL-4 responses against GPI- as well as non-GPI-anchored forms of PA, respectively. $CD4^+$ T cells generated IL-4 responses exclusively against the non-GPI-anchored form of PA. Also, MHC I-restricted responses had no role to play. As a result, the group pTPA.GPI-PA mounted combined CD1d- and MHC II- restricted IL-4 responses whereas the group pGPI-PA and pPA-Native mounted exclusively CD1d-restricted and MHC-II restricted responses, respectively.

3.5. CD1d- and MHC II-restricted help to B-cells aided IgG formation. $CD4^+NK1.1^+$ helper T cells provide help to B cells to support Ig formation [36]. To assess this we co-cultured autologous B lymphocytes with $CD4^+NK1.1^+$ helper T cells in the presence of GPI-attached PA peptides (Fig.3). As expected, NKT cells isolated from the group pTPA.GPI-PA and pGPI-PA extended efficient help to B lymphocytes for IgG formation (Panel A & E, Fig.3). This response was also completely dependent upon CD1d-restriction of the GPI-attached PA as high SFUs (Spot Forming Units) were obtained only in the absence of anti-CD1d antibody. However, such a response was slightly higher for the group pGPI-PA which developed SFU$\geq$ 600 as compared to the group pTPA.GPI-PA which also displayed a high SFU of $\leq$600 (Panel A & E). Importantly, the group pTPA.GPI-PA also displayed MHC II-restricted NKT cell mediated IgG responses against non-GPI PA peptides (Panel F) as opposed to only CD1d-restricted responses seen for the group pGPI-PA (Panel A & B). In addition to this, $CD4^+$ T cell-mediated B cell helper responses to non-GPI PA peptides were also evaluated. These results were in corroboration with those obtained for IL-4 production and also detailed the generation of MHC II-restricted IgG responses to non-GPI PA peptides, by both the group pTPA.GPI-PA and pPA-native (Panel H & L). Overall results pointed the development of dual CD1d- and MHC II-restricted $CD4^+NK1.1^+$ helper T responses as well as MHC-II restricted $CD4^+$ T cell responses for the group pTPA.GPI-PA as opposed to the group pGPI-PA which displayed CD1d-restricted NKT cell helper responses exclusively.

3.6. Correlation between anti-PA/Letx neutralization titers and protection against toxin challenge. Immunized mice were challenged with a Letx mixture (50μg PA and 22 μg LF, ≈4-5 LD_{50}) in a total volume of 100 μl via tail vein injection at different time intervals (12, 14, 16, 18 & 20 weeks) post last immunization. Toxin challenge results as obtained are summarized in Table 3. Control mice (vector immunized) died after receiving an anthrax lethal toxin injection with a MTD (Mean Time to Death) around 0.7 ± 0.2 days. All mice immunized with pTPA.GPI-PA resisted toxin challenge and elicited a survival percentage of 100% at all the time intervals post immunization (Table3). The average neutralization titers for this group were $\geq 10^3$ and anti-PA titers were $\geq 10^5$. On the other hand, the group pGPI-PA showed a 100% survival till week 16 with average neutralization titers for protection ranging between 600-800. Letx neutralization titers below this range (400-500) conferred protection to 75-80% of the mice and increased MTD to 8.5-10.4 days. Results also depicted that both anti-PA and Letx neutralization titers leveled off at titers about $\geq 10^5$ and $\geq 10^3$ respectively, for the group pTPA.GPI-PA; and ≥80,000 and 450 respectively, for the group pGPI-PA post week 14. For the group pPA-Native, neutralization titers ranging from 100-300 conferred protection to 45-55% of the mice with an average MTD ranging from 3.3 to 5.9 days.

Statistical differences between the delays in time-to-death as measured by log rank statistic for the groups immunized with GPI-bearing chimeras compared to the vector control group showed that the differences were quite significant ($p<0.01$) and correlated well with survival (r=0.85). Analysis of post-challenge TNA titers showed that survival at week 14 and 16 correlated well with each other (r=0.89). The results therefore, suggested that mice immunized with DNA vaccine chimeras bearing GPI-anchor developed significant Letx neutralization titers which protected the mice against anthrax lethal toxin challenge. Importantly, DNA immunization alone with the chimera pTPA.GPI-PA provided 100% protection to animals till week 20 post priming.

3.7. High-avidity PA-specific Abs were generated at the time of challenge. Avidity of the antibodies generated against PA was determined by urea-based ELISA described in the materials and methods. Avidity index (AI) of the antibodies generated was ≤ 40 in all animals 2 weeks after the second boost (Table 5). Animals inoculated with pPA-Native chimera had the lowest avidity index of 23. Following anthrax lethal toxin challenge, intermediate to high avidity antibodies were generated by the immunized mice. Group immunized with pTPA.GPI-PA and pGPI-PA showed higher avidity indices of 79 and 62 respectively, whereas the group pPA-native showed a significantly lower AI value of 34. Four weeks post challenge, a further increase in AI was observed for all the groups (Table 5) with groups pTPA.GPI-PA and pGPI-PA showing avidities as high as 87 and 78 respectively, as opposed to an AI=53 for the pPA-Native group.

4. DISCUSSION

Although the currently licensed anthrax vaccine (AVA) generates potential anti-anthrax immunity, it suffers from drawbacks like reactogenicity and long dosage schedule with an additional need for frequent administration of boosters. Clearly, there is reason to explore alternatives. Therefore, based on the hypothesis that GPI-anchored form of PA will improve the DNA vaccination against anthrax, we constructed a DNA chimera encoding the protease-cleaved fragment of protective antigen (PA63) attached to a C-terminal mammalian PLAP (Placental Alkaline Phosphatase) GPI anchor sequence. Along with that we constructed another chimera bearing PA63 gene with an N-terminal TPA leader alongwith a C-terminal PLAP-GPI anchor.

In vitro expression of the chimeras in the J774A.1 mouse MΦ-like cells was evaluated to test whether the gene for PA and the signals were being properly recognized in the mammalian cells. Subcellular fractionation and western blotting indicated that the PA protein along with the GPI and TPA signal was being properly recognized by the mammalian cells. The chimeras, pGPI-PA and pTPA.GPI-PA, expressed membrane-anchored form of PA. In addition, the chimera pTPA.GPI-PA also mediated the secretion of PA in the cell culture supernatants.

Earlier studies have underscored the fact that anti-PA antibodies have antispore activity and might have a role in impeding the early stages of infection with *B. anthracis* spores [54]. Also, passive transfer of anti-PA antibodies has been shown to provide protection against anthrax infection in guinea pigs [55]. Therefore, to test the ability of GPI anchored PA-expressing chimeras to generate protective humoral responses in vivo, we injected mice with the DNA vaccine chimeras and determined the anti-PA reactivity in the serum of the immunized animals. All vaccinated animals generated potential IgM titers after the first inoculation. These titers declined after the first and second booster suggesting successful priming. Remarkable serum IgG antibody titers were observed after the third immunization for both the constructs encoding GPI-anchored form of PA. These responses were approximately two to three times higher than those generated by pPA-Native chimera indicating that GPI-anchored forms evoked better PA-specific humoral responses than the non-GPI anchored form of PA. Importantly, electron microscopic studies have shown that spore-associated proteins can be recognized by anti-PA antibodies, and PA-immune serum from several species enhanced the phagocytosis of spores of the virulent Ames and Sterne strains by murine peritoneal macrophages [54, 56, 58]. In addition, it has been shown that IgG subclass displays highest affinity towards PA [57]. Therefore, development of high end-point titers of IgG antibody upon DNA vaccination gains high merit from anti-spore activity point of view.

Anthrax Letx neutralization titers were also analyzed to assess protection against massive toxemia and septicemia caused by *Bacillus anthracis* tripartite toxin. Sera from the vaccinated animals demonstrated Letx neutralizing activity only after the first booster dose. The mean TNA titers were found to be higher for the groups that received chimeras encoding GPI-anchored form of PA. Peak toxin neutralizing titers appeared at week 14 post priming. Anti-PA titers of $\geq 10^5$ and neutralization titers $\geq 10^3$ resisted challenge and conferred 100% protection to the animals in the group pTPA.GPI-PA. Earlier studies with guinea pigs [59, 60] and rabbits [61] have shown that anti-PA titers $\geq 10^5$ and ≥ 300, respectively, confer 100% protection. Thus, minimum protective anti-PA antibody titers have been found to differ depending upon variables of the host [62].

Neutralization titers after attaining a peak at week 14, first declined and then plateaued. This decline in neutralization titer paralleled the declining survival percentage supporting the notion that antibodies involved in neutralization are the ones that afford protection against Letx challenge. This was also shown by the increased delay in death in partially protected mice as compared to the control mice. Statistical differences between the delays in time-to-death as measured by log rank statistic for the groups immunized with GPI-bearing chimeras compared to the vector control group showed that the differences were quite significant ($p<0.01$) and correlated well with survival (r=0.85). Further, survival at week 14 and 16 correlated well with the TNA titers developed at these two time points (r=0.89). Such a correlation between protection and neutralization titers has also been observed in other animal models like guinea pigs [59, 60] and rabbits [61] suggesting that this phenomenon is not species-specific. Further on subsequent immunizations, there was progressive increase in the avidity of the antibodies. Investigation of memory responses in terms of affinity maturation of antibodies (antibody avidity) upon challenge indicated that a challenge engendered amanestic immune response was mounted. Taken together, these Letx neutralization antibodies and anti-PA antibodies generated upon immunization with the GPI-anchored forms of PA can efficiently confer protection against both toxemia and bacteremia; and can therefore, provide an armour against infection.

To examine the fine specificity of the T-cell subsets generated upon DNA vaccination we examined the in vitro proliferation of the splenocytes in the presence of GPI-attached- as well as non-GPI-PA peptides which revealed that $CD4^+NK1.1^+$ helper T cells were the main target for activation by GPI-anchored PA. These results were consistent with the seminal study which illustrated that cellular GPI and glycolipids are natural ligands of mouse CD1d, a member of CD1 family of evolutionarily conserved MHC-like molecules [63]. These CD1d molecules in turn have been shown to directly control the function of $NK1^+$ natural killer T (NKT) cells, a heterogenous subset of T cells displaying a $CD4^+$ or $CD4^-CD8^-$ double negative phenotype, and co-expressing the natural killer cell receptor NK1.1/ NKR P1A and a semi-invariant TCR encoded in mice [64]. Interestingly, an appreciable population of NKT cells also resulted from proliferation, in the presence of non-GPI PA peptides, from the group pTPA.GPI-PA indicating that NKT cells isolated from this group responded well against both GPI as well non-GPI anchored form of PA. However, NKT cells clonally expanded to relatively higher numbers in the presence of GPI-attached PA peptides as compared to non-GPI-associated PA peptides. Results therefore, point that somehow the posttranslational modifications of the expressed antigen in the mammalian systems in vivo affected their ability to activate innate immunity.

Generation of Ig responses against an antigen is as a result of classical MHC-II-dependent "cognate-help" which requires BcR-mediated antigen internalization and presentation of specific epitopes to antigen-specific T cells [65]. Studies however, suggest a direct role for invariant $CD4^+$ NKT cells in regulating CD1d-restricted B cell help which regulates B lymphocyte proliferation and effector functions [66]. Therefore, to establish the restriction requirements of this NKT cell lineage, we evaluated the IL-4 responses. We show here that the recognition of GPI-anchored PA by the NKT cells (from groups pGPI-PA and pTPA.GPI-PA) is MHC-independent and CD1-restricted. However, analysis of similar responses by $CD4^+$ helper T cells isolated from the group pTPA.GPI-

PA and pPA-Native indicated that these responses were on account of MHC II-mediated restriction of non-GPI-attached PA. Interestingly, NKT cells from the group pTPA.GPI-PA displayed MHC II-restriction and CD1-independence when presented with the non-GPI PA peptides.

B-cell helper responses generated by $CD4^+NK1.1^+$ helper T cells also paralleled those generated for IL-4 production. The results therefore, gave direct evidence that the NKT cells from the group pGPI-PA extended purely CD1d-restricted help whereas those from the group pTPA.GPI-PA mediated both CD1d- as well as MHC-II restricted B-cell help. In addition, the group pTPA.GPI-PA also gave rise to $CD4^+$ helper T cell population which mediated effector responses that were dependent on classical MHC II restriction of non-GPI anchored PA. All these factors contributed to the heightened IL-4- and B-cell helper-responses obtained for the group pTPA.GPI-PA as compared to those obtained for the group pGPI-PA.

There are reports that have shown that GPI-anchored proteins occur as microdomains in cell membrane in living cells [67]. In this manner the alkyl acyl chain of the GPI-anchored proteins can make contacts with the CD1 pocket on the cell surface on account of the unique capacity of CD1 molecules to bind and exchange lipids also on the cell surface [68]. Therefore, generation of predominantly CD1d-restricted NKT cell responses by the group pGPI-PA and pTPA.GPI-PA could probably be as a result of the cell surface recognition of membrane-anchored PA by CD1d molecules. However, the NKT cells and $CD4^+$ helper T cells isolated from the group pTPA.GPI-PA also displayed Ig responses to non-GPI anchored PA that were MHC-II restricted and CD1-independent which suggested that the antigen expressed by this chimera was being channeled to MHC II pathway as well. This could possibly be as a result of deacylation of the expressed GPI-anchored antigen either in ER or upon secretion by endogenous host phospholipases.

TPA is a protein that is expressed by vascular smooth muscle and binds to a specific cellular receptor p63, occurs in fibroblasts as an intracellular protein associated with the ER [42, 43]. The TPA leader peptide possibly mediated targeting of the expressed antigen directly to the ER. As matter of fact, TPA leader peptide has also been shown to mediate the secretion of the proteins attached to them [69-71]. This was also seen in our case following in vitro transfection and immunoblot analysis. This secreted (deacylated) protein was channeled to MHC II pathway as indicated by abrogation of effector responses generated by NKT cells and $CD4^+$T helper T cells upon addition anti-MHC II mAb (Fig. 2 & 3). Overall, there was successful antigen trafficking to both pathways of antigen presentation that is CD1 as well as MHC II in response to GPI-anchored and non-GPI anchored forms of PA, respectively.

To our knowledge there is no published work demonstrating the development of NKT cell mediated helper responses generated by combined CD1d- and MHC II-restriction of the antigen delivered via i.m DNA vaccination. Responses generated against the GPI-anchored form of PA specifically mediated the maturation and rapid proliferation of $CD4^+NK1.1^+$ helper T cell subset which rapidly produced IL-4 in response to T cell receptor ligation. These cells were CD1d reactive and upon antigen recognition responded *en masse*. Therefore, the first and foremost advantage of our vaccination strategy was that we successfully generated CD1 restricted responses whereby the chief player i.e. CD1 molecule shows minimal allelic polymorphism. Also, mouse and human CD1d molecules are widely broadly distributed on most cells of hematopoietic origin, with the highest levels observed on leukocytes such as dendritic cells, B cells or monocytes that will assure constant immune surveillance. Additional help provided by the classical MHC II restricted NKT- and $CD4^+$ T-helper cell responses further resulted in the augmentation of useful responses. Therefore, successful targeting to the non-conventional CD1 pathway alongwith the classical MHC II pathway can be devised as a useful strategy for generating heightened humoral immune responses against antigens. We successfully illustrate here the generation of efficient humoral responses against anthrax protective antigen that generated protective immunity against anthrax lethal toxin challenge. Further work can gain a complete understanding regarding the immune pathways involved which can ultimately lead to the design and development

of better human vaccines against anthrax to counter emergency.

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Table 1. Sequence of the peptides used for in vitro cell stimulation assays.

SEQ ID	SEQUENCE	REFERENCE
PA 64-77	IWSGFIKVKKSDEY	72
PA 112-127	RLYQIKIQYQRENPTE	73
PA 154-167	PELKQKSSNSRKKR	72
PA 547-560	ITEFDFNFDQQTSQ	72
PA 659-672	RYDMLNISSLRQDG	72
PA 717-730	STNGIKKILIFSKK	72
GPI-anchor	GTTDAAHPGRSVVPALLPLLAGTLLLLETATAP	74

Table 2. Anti-PA antibody titers in the serum of DNA immunized animals.

	Antibody end point titers [a], 10^3 ±SD								
	IgM			IgG			Letx Neutralizing antibody		
Construct	Priming	I[st] Boost	2[nd] Boost	Priming	I[st] Boost	2[nd] Boost	Priming	I[st] Boost	2[nd] Boost
pGPI-PA	0.48±0.1	<0.05	<0.05	25.0±4.0	47.0±6.5[b,c]	79.0±4.1[b,c]	-BD	0.35±0.11[b]	0.51±0.2[b]
pTPA.GPI-PA	0.55±0.2	<0.05	<0.05	13.5±1.5	88.0±5.5[b,c]	100±4.0[b,c]	-BD	0.75±0.23[b]	1.0±0.15[b]
pPA-Native	0.37±0.2	<0.05	<0.05	3.5±0.5	10.0±2.0	20±2.1	-BD	0.10±0.01	0.15±0.1
Vector control	-BD[d]	-BD	-BD	-BD	-BD	-BD	-BD	-BD	-BD

[a] Mice were immunized at days 0, 28 and 56; and anti-PA titers were measured in the serum of the immunized mice 14 days after each immunization. Results represent titers obtained for 8-10 mice tested in a group in three independent experiments and results are expressed as mean ±SE.

[b] Significantly higher ($p<0.001$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c] Significantly higher ($p<0.01$) titers as compared to those developed after priming as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d] Below detection.

Table 3. Correlation of protection against B.anthracis lethal toxin challenge and anti PA-/ Letx neutralization antibody titers at different time points post vaccination.

Group	Time of challenge [a]	ANTIBODY TITERS POSTCHALLENGE, 10^3±SD: Total Anti-PA Antibody	Letx Neutralizing Antibody	Survival% (No.of survivors/ No. challenged)	MTD[b]±SD (Days)
pGPI-PA	12	85.5±5.5	0.65±0.20	100 (9/9)	9.5±0.9[C]
	14	91.4±8.8[C]	0.75±0.10[C]	100 (8/8)	
	16	90.5±7.0[C]	0.70+0.20[C]	100 (7/7)	
	18	80.1±6.5	0.49±0.15	75 (6/8)	9.5±0.5[C]
	20	80.5±7.5	0.45±0.12	75 (5/7)	8.9±0.4
pTPA.GPI-PA	12	120±5.5	1.30±0.50	100 (8/8)	
	14	199±6.0[C]	1.95±0.40[d]	100 (8/8)	
	16	175±8.0[C]	1.70±0.20[d]	100 (5/5)	
	18	155±3.0	1.55±0.15	100 (7/7)	
	20	140±2.0	1.51±0.11	100 (8/8)	
pPA-Native	12	26.0±4.0	0.19±0.05	43 (3/7)	3.6±0.6
	14	41.0±6.5	0.25±0.02	56 (5/9)	5.9±0.2
	16	34.0±3.5	0.24±0.03	56 (5/9)	5.7±0.5
	18	20.0±2.5	0.19±0.03	44 (4/9)	3.6±0.5
	20	19.0±2.5	0.18±0.05	44 (4/9)	3.3±0.6
Vector control	12	-BD [e]	-BD	0 (0/5)	0.70±0.2
	14	-BD	-BD	0 (0/4)	0.70±0.2
	16	-BD	-BD	0 (0/4)	0.70±0.2
	18	-BD	-BD	0 (0/5)	0.70±0.2
	20	-BD	-BD	0 (0/6)	0.70±0.2

[a] The immunized mice were challenged at 12, 14, 16, 18, 20 weeks post immunization with anthrax lethal toxin mixture of PA (50 μg/mouse) and LF (22 μg/mouse) injected i.v. through tail vein and monitored for 15 days. Results represent titers obtained for n=9 mice tested in a group in three independent experiments and results are expressed as mean ±SE.

[b] The mean time to death (MTD) was determined by determining the average time to death for each group, excluding the survivors. Represented as mean ±SE.

[c] Significantly higher ($p<0.01$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d] Significantly higher ($p<0.001$) titers as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[e] BD, below detection.

Table 4. Specific phenotype analysis of effector cells by flow cytometry.

	Cell proliferation [a] in the presence of GPI-PA		Non-GPI-PA	
Construct	$CD3^+CD4^+$ (%)	$CD4^+NK1.1^+$ (%)	$CD3^+CD4^+$ (%)	$CD4^+NK1.1^+$ (%)
pGPI-PA	5.0	75.7 [b]	10.0	19.5
pTPA.GPI-PA	18.7	71.5 [b]	45.3 [c]	39.7 [c]
pPA-Native	9.6	5.0	55.7	8.6

[a] Splenocytes from naive or DNA vaccinated mice (n=8-10) were cultured in the presence of pooled PA-derived peptides [either GPI-attached or non-GPI- PA peptides (10μg/ml of each)] with IL-2 (10 U/ml) over a period of 4 days. Thereafter, $NK1.1^+TCR\text{-}\alpha/\beta^+$ cells and $CD4^+$ T cells were analyzed by flow cytometry.

[b] Significantly higher ($p<0.001$) percentage as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c] Significantly higher ($p<0.05$) percentage as compared to the pGPI-PA group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

Table 5. Avidity indices of anti-PA antibodies in the serum of immunized mice.

Construct	Antibody avidity index [a]		
	Day 70	Day 100 Challenge	Day 28 Post-challenge
pGPI-PA	33	62 [b, c]	78 [b, c]
pTPA.GPI-PA	39	79 [b, c]	87 [b, c]
pPA-Native	23	34	53
Vector control	-BD [d]	-BD	-BD

[a] Avidity index= (endpoint titer in the presence of urea)/ (endpoint titer in the absence of urea)) x 100.

[b] Significantly higher ($p<0.001$) avidity index as compared to the pPA-Native group as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[c] Significantly higher ($p<0.01$) AI value as compared the one at Day 70 as calculated by Student's t-test followed by Bonferroni's correction to adjust for multiple comparisons.

[d] No PA-specific antibody was detected.

CAPTIONS:

FIG. 1. The address tags efficiently target PA63 to various sub cellular locations. Shows expression of different forms of protease-cleaved fragment of protective antigen in J774.A1 mouse MΦ-like cells. Cells were transfected with DNA vaccine constructs. Cell lysates and cell membrane fractions were prepared 48 hrs post-transfection. Alongwith that cell culture proteins were harvested by acetone precipitation. Subsequently, the protein samples were subjected to 12% SDS-PAGE under reducing conditions and transferred onto a nitrocellulose membrane. Blot was probed with anti-PA polyclonal serum followed by alkaline phosphatase-conjugated donkey anti-rabbit IgG and developed using BCIP/NBT as substrate.

FIG.2. Combined CD1d- and MHC II-restricted IL-4 responses were generated pTPA.GPI-PA chimera. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized to take out the spleens and spleen cells were were used for the isolation of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. NKT cells and $CD4^+$ helper T cells were cultured in the presence 10μg/ml GPI-PA and non-GPI-PA to determine the cytokine secretion in vitro. Cell-free supernatants were collected 24 h. Each bar represents the concentration of IL-4 secreted by NKT cells/$CD4^+$ T cells from 7-8 mice in a group tested in three different experiments and error bars represent the ±SEM. (The error bars represent 95% confidence intervals calculated from the group wise mean concentration. *, **, ***, and ****, Significant difference of the response relative to pPA-Native immunization defined as p <0.0001, p <0.001, p <0.01and p<0.05, respectively).

FIG.3. CD1d- and MHC II-restricted help to B-cells aided IgG formation. Mice were immunized i.m. with different DNA vaccine constructs. At day 70 mice were euthanized to take out the spleens and spleen cells were were used for the isolation of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ T cells by cell sorting using FACS Vantage^{TM} instrument. IgG ELISPOT analysis of $CD4^+$ $NK1.1^+$ helper T cells and $CD4^+$ helper T cells in response to GPI-PA and non-GPI-PA day 70 (n=5–6/group). ELISPOT data is expressed as the calculated standard error mean (± SEM) of the Ag-stimulated cells minus un-stimulated cells. Each bar represents number of SFC's obtained for 7-8 mice in a group tested in three different experiments and error bars represent the ±SEM. (The error bars represent 95% confidence intervals calculated from the group wise mean SFC. *, **, ***, and ****, Significant difference of the response relative to pPA-Native immunization defined as p <0.0001, p <0.001, p <0.01and p<0.05, respectively.)

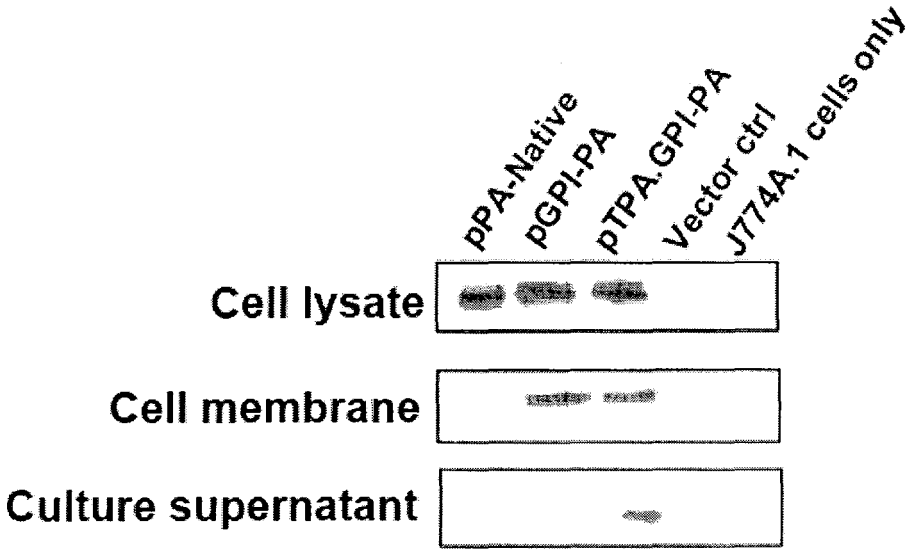
pPA-Native
pGPI-PA
pTPA.GPI-PA
Vector ctrl
J774A.1 cells only
Cell lysate
Cell membrane
Culture supernatant

Fig.1

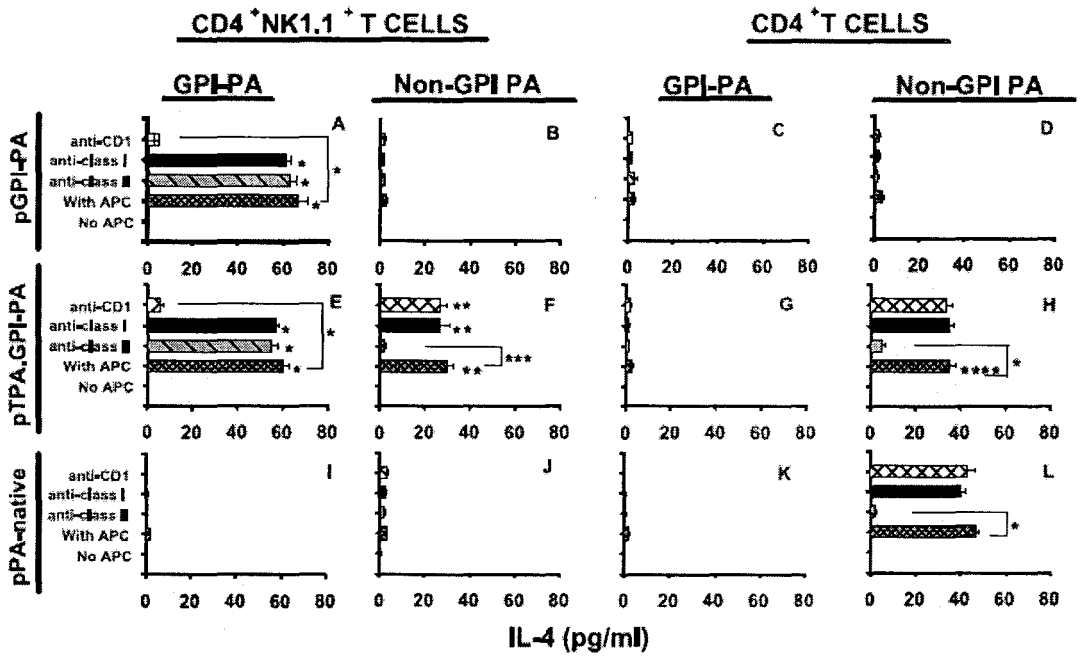
CD4+NK1.1+ T CELLS
CD4+T CELLS
GPI-PA
Non-GPI PA
GPI-PA
Non-GPI PA
pGPI-PA
pTPA.GPI-PA
pPA-native
anti-CD1
anti-class I
anti-class II
With APC
No APC
A
B
C
D
E
F
G
H
I
J
K
L
0 20 40 60 80
IL-4 (pg/ml)

Fig.2

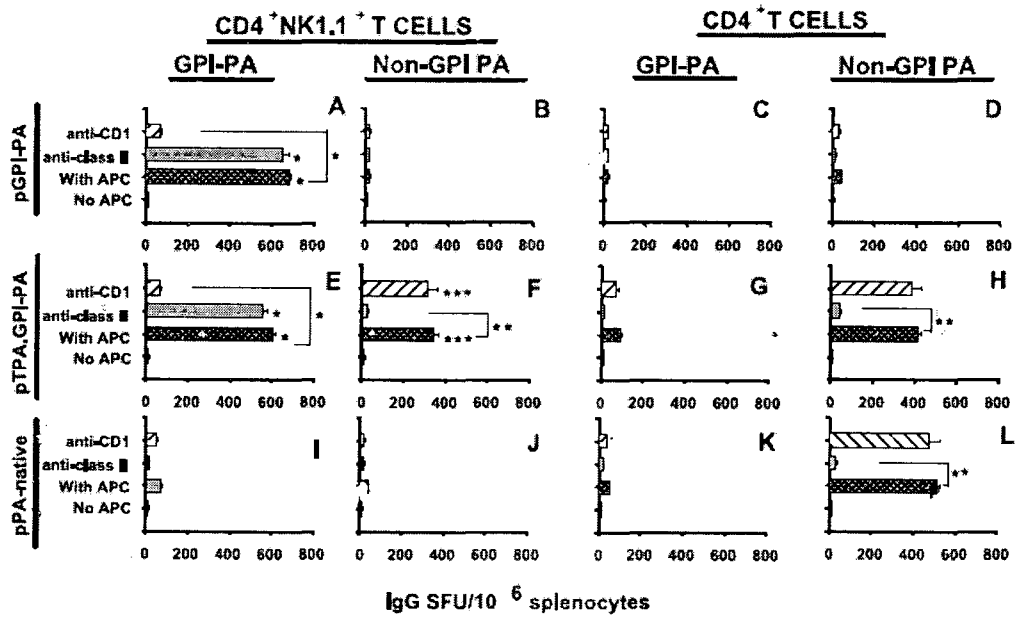
CD4+NK1.1+ T CELLS
CD4+T CELLS
GPI-PA
Non-GPI PA
GPI-PA
Non-GPI PA
pGPI-PA
pTPA.GPI-PA
pPA-native
anti-CD1
anti-class II
With APC
No APC
A
B
C
D
E
F
G
H
I
J
K
L
0 200 400 600 800
IgG SFU/10 6 splenocytes

Fig.3

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Cloning, expression, and characterization of recombinant nitric oxide synthase-like protein from *Bacillus anthracis* ☆

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Abstract

Nitric oxide synthase (NOS) is amongst a family of evolutionarily conserved enzymes, involved in a multi-turnover process that results in NO as a product. The significant role of NO in various pathological and physiological processes has created an interest in this enzyme from several perspectives. This study describes for the first time, cloning and expression of a NOS-like protein, baNOS, from *Bacillus anthracis*, a pathogenic bacterium responsible for causing anthrax. baNOS was expressed in *Escherichia coli* as a soluble and catalytically active enzyme. Homology models generated for baNOS indicated that the key structural features that are involved in the substrate and active site interaction have been highly conserved. Further, the behavior of baNOS in terms of heme–substrate interactions and heme-transitions was studied in detail. The optical perturbation spectra of the heme domain demonstrated that the ligands perturb the heme site in a ligand specific manner. baNOS forms a five-coordinate, high-spin complex with L-arginine analogs and a six-coordinate low-spin complex with inhibitor imidazole. Studies indicated that the binding of L-arginine, N^{ω}-hydroxy-L-arginine, and imidazole produces various spectroscopic species that closely correspond to the equivalent complexes of mammalian NOS. The values of spectral binding constants further corroborated these results. The overall conservation of the key structural features and the correlation of heme–substrate interactions in baNOS and mammalian NOS, thus, point towards an interesting phenomenon of convergent evolution. Importantly, the NO generated by NOS of mammalian macrophages plays a potent role in antimicrobicidal activity. Because of the existence of high structural and behavioral similarity between mammalian NOS and baNOS, we propose that NO produced by *B. anthracis* may also have a pivotal pathophysiological role in anthrax infection. Therefore, this first report of characterization of a NOS-like protein from a pathogenic bacterium opens up avenues for further studies in understanding the importance of this protein in pathogenicity.

Keywords: *Bacillus anthracis*; Nitric oxide synthase; Optical spectral perturbation; Heme–substrate interaction

Nitric oxide synthase (NOS) is a family of bimodal enzymes, comprising of an N-terminal oxygenase domain (NOSoxy) that binds protoporphyrin IX (heme), 6*R*-tetrahydrobiopterin (H_4B), and L-arginine (Arg); and a C-terminal reductase domain (NOSred) that binds FMN, FAD, and NADPH [1]. The two domains are linked together by a calmodulin (CaM) binding sequence [1]. CaM binding activates NO synthesis by triggering electron transfer from the flavins to heme [2]. Sequential transfer of electrons, thus, enables the heme to bind and activate oxygen in both steps of NO synthesis, resulting in NADPH- and

☆ *Abbreviations:* NOS, nitric oxide synthase; baNOS, *Bacillus anthracis* nitric oxide synthase; deiNOS, *Deinococcus radiodurans* nitric oxide synthase; bsNOS, *Bacillus subtilis* nitric oxide synthase; NOSoxy, nitric oxide synthase oxygenase domain; NOSred, nitric oxide synthase reductase domain; H_4B, (6*R*)-5,6,7,8-tetrahydrobiopterin; Ni–NTA, Ni $^{2+}$–nitrilotriacetic acid; DTT, dithiothreitol; EPPS, (*N*-[2-hydroxyethyl]piperazine-*N*′-[3-propanesulfonic acid]; HEPPS); BCIP, 5-bromo-4-chloro-3-indolyl phosphate disodium salt; NBT, nitro blue tetrazolium; PA, protective antigen; LF, lethal factor; EF, edema factor.

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Oral vaccines: new needs, new possibilities

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Summary

Vaccination is an important tool for handling healthcare programs both in developed and developing countries. The current global scenario calls for a more-efficacious, acceptable, cost-effective and reliable method of immunization for many fatal diseases. It is hoped that the adoption of oral vaccines will help to provide an effective vaccination strategy, especially in developing countries. Mucosal immunity generated by oral vaccines can serve as a strong first line of defense against most of the pathogens infecting through the mucosal lining. Advances in elucidating the mechanism of action of oral vaccines will facilitate the design of more effective, new generation vaccines. There are promising developments in the use of different agents to effectively deliver the vaccine candidate. It is hoped that ongoing research may be able to set another cardinal point, after polio vaccine, in eradicating infectious diseases. ***BioEssays*** **29:591–604, 2007. © 2007 Wiley Periodicals, Inc.**

Introduction

In spite of advances in the scientific knowledge of infectious diseases, the health-care situation remains grim due to several factors, Social and demographic structure along with the economic status of a particular region are important points of consideration when designing health-care programs especially in developing countries. One very efficient and tenable strategy is based on the age-old adage "Prevention is better than cure". It was the concept of vaccination that heralded the era of a planned health management.

About 15 million (>25%) of 57 million annual deaths worldwide are estimated to be related directly to infectious diseases.[1] Successful implementation of vaccination programs has helped to contain infectious diseases and has led to complete eradication in certain cases. In the last 5–10 years, many new vaccine strategies have been designed based on a substantial increase in fundamental knowledge of the immune system, and the recognition of better vaccine candidates. Some of these vaccines have advanced to clinical trials.[2] Some countries have experienced the benefits of planned immunization and have been successful in protecting infants against ten lethal infectious diseases. The implementation of similar programs in developing countries has led to the protection of 75% infants against a subset of six of these diseases, diphtheria, pertussis, tuberculosis, measles, tetanus and polio.[3]

The pathogens responsible for the greatest burden of human diseases make initial contact with the human host at a mucosal site such as the respiratory tract, gastrointestinal tract or genitourinary tract.[4] Invasion by these pathogens can be effectively prevented by the stimulation of an immune response of suitable strength and quality at these mucosal surfaces. Efforts in this direction have led to the development of oral vaccines.

Oral vaccines (Table 1) are relatively new in the arena of health-care instruments. These vaccines have been found to be promising in generating mucosal immunity. Two oral vaccines, the Sabin Oral polio vaccine and Ty21 typhoid vaccine, are included in vaccination programs in USA.[5] The most-successful and widely used Sabin polio vaccine was adopted in 1960s. The successful use of this vaccine in eradicating polio from several countries led to extensive research in the area of oral vaccines. This review highlights the potential of oral vaccines as important healthcare tools, especially in developing countries. The mechanism of action of oral vaccines is discussed along with different modes of administration. The status of oral vaccines against important diseases is mentioned followed by the current limitations, which may provide insights for improving the performance of these vaccines.

Concerns related to parenteral vaccines

Though the concept of vaccination is centuries old and several diseases have been conquered through the successful implementation of vaccines as health-care tools, we are still witnessing almost 3 million infant deaths due to completely

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Evaluation of the ability of N-terminal fragment of lethal factor of *Bacillus anthracis* for delivery of *Mycobacterium* T cell antigen ESAT-6 into cytosol of antigen presenting cells to elicit effective cytotoxic T lymphocyte response

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Abstract

We report the ability of N-terminal fragment of lethal factor of *Bacillus anthracis* to deliver genetically fused ESAT-6 (early secretory antigen target), a potent T cell antigen of *Mycobacterium tuberculosis*, into cytosol to elicit Cytotoxic T lymphocyte (CTL) response. *In vitro* Th1 cytokines data and CTL assay proved that efficient delivery of LFn.ESAT-6 occurs in cytosol, in the presence of protective antigen (PA), and leads to generation of effective CTL response. Since CTL response is essential for protection against intracellular pathogens and, it is well known that only single T cell epitope or single antigenic protein is not sufficient to elicit protective CTL response due to variation or polymorphism in MHC-I alleles among the individuals, we suggest that as a fusion protein LFn can be used to deliver multiepitopes of T cells or multiproteins which can generate effective CTLs against intracellular pathogens like *M. tuberculosis*. It can be used to enhance the protective efficacy of BCG vaccine.

Keywords: Fusion; CTL; Cytokines; Lethal factor

Bacillus anthracis produces a bipartite A/B-type toxin. The B subunit is the 83-kDa protective antigen (PA) receptor-binding moiety (named for its use as a vaccine), and the two catalytic A subunit moieties are edema factor (EF; 89 kDa) and lethal factor (LF; 90 kDa). EF is a Ca^{2+}- and calmodulin-dependent adenylate cyclase [1]. LF is a Zn^{2+}protease that cleaves and inactivates mitogen-activated protein kinase kinase-1 and -2 [2]. PA binds to integrin-like I domain of anthrax toxin receptor/tumor endothelial marker 8 (ATR/TEM8) or the capillary morphogenesis protein2 (CMG2) receptor on the cell surface [3], gets activated by furin family protease to a 63-kDa fragment (PA63). PA63 forms ring-shaped homoheptamer prepore $(PA63)_7$ which can bind up to three molecules of EF or LF or both [4,5] to form $(PA63)_7$-LF/EF which undergoes clathrin-dependent endocytosis, and LF or EF is released to the cytosol through homoheptamer prepore in a pH-dependent manner [4,6] where they exert their cytopathic effects.

N-terminal PA binding region (LFn) of lethal factor has been used to deliver heterologous protein and peptide into cytosol and to process it by MHC class I antigen presentation pathway and to elicit CTL response [7–9]. However, it has been reported that some proteins fused to LFn are not translocated in cytosol via PA [10] and CTLs are very

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Induction of cytotoxic T lymphocyte response against Mycobacterial antigen using domain I of anthrax edema factor as antigen delivery system

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Abstract

We have investigated the efficiency of N-terminal 1–260 residues of Edema factor (EFn) as a delivery system for ESAT-6, an antigenic protein of *Mycobacterium tuberculosis* $H_{37}R_v$, into the cytosol of mammalian cells. The EFn.ESAT-6 recombinant protein was obtained by genetic fusion of EFn and ESAT-6 DNA. Our data shows that in the presence of PA, EFn.ESAT-6 fusion protein is internalized into the cytosol of antigen presenting cells, and the splenocytes produced both Th1 and Th2 cytokines *in vitro*. Further, EFn.ESAT-6 elicited effective cytotoxic T lymphocyte (CTL) response in an *in vitro* CTL assay. This study for the first time demonstrates that EFn can be used as a vehicle to deliver heterologous proteins of therapeutic importance.

Keywords: Edema factor; ESAT-6; Fusion protein; CTL; Cytokines

Humoral and cellular responses constitute the two main arms of immune system. One of the key functions of cellular immunity is to generate cytotoxic T lymphocytes (CTLs) for destruction of cells expressing intracellularly processed antigens on their surface. The CTLs recognize and kill tumor and other diseased cells, which display non-self peptides on their surface [1,2]. These peptides arise from various sources, such as infectious agents or aberrant expression of self-proteins, and mark defective cells for CTL recognition. Proteins within the cytosol are processed by multi-catalytic proteosome to generate small peptides, which are then displayed by class I major histocompatibility molecules (MHC-I) on the cell surface. Recognition of foreign peptide–MHC-I complexes by $CD8^+$ cells leads to activation of specific CTLs, which clear the defective cells expressing foreign peptides or harboring pathogen [1,2–5]. Activated CTLs lyse infected cell, secrete cytokines, proliferate and differentiate. Vaccines that prime such memory CTLs, provide protection to the host, upon subsequent exposure to similar antigen displaying cells [5].

Development of vaccines with the ability to generate specific CTLs is hindered due to paucity of delivery systems of antigenic CTL epitopes into the cytosol of host cells. Several approaches to this problem have been reported [1] including the use of attenuated viruses, intracellular bacteria, bacterial toxins, naked DNA, electroporation, heat shock protein, polycationic peptides, non-ionic triblock copolymer, and adjuvants [6–11]. Each of these methods have inherent problem of safety and/or efficiency. Non-infectious, non-toxic, modified bacterial toxins for delivery of heterologous proteins have been reported in the past [6,8,12–17].

Bacillus anthracis produces a bipartite exotoxin comprising of two toxins namely edema toxin (ET) and lethal toxin (LT). Both ET and LT contain a protective antigen (PA) component along with either edema factor (EF) or lethal factor (LF), respectively. Individually, PA, EF, and LF are non-toxic. PA mediates entry of EF and LF into the

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