DEVELOPMENT OF A MUCOSAL DNA VACCINE FOR EQUINE INFLUENZA

By

SHEELA RAMAMOORTHY

Bachelor of Veterinary Science

Madras Veterinary College

Madras, India

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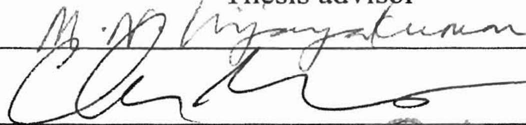

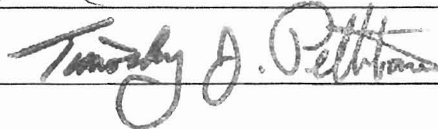

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NOMENCLATURE

ADCC	Antibody dependent cytotoxicity
ATCC	American type tissue culture collection
BCIP	5-Bromo-4-Chloro-3-Indolyl phosphate
BSA	Bovine serum albumin
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CTL	Cytotoxic T Lymphocytes
DMEM	Dulbecco's modified Eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetracetic acid
EH	Equine hemagglutinin
ELISA	Enzyme linked immunosorbent assay
FBS	Fetal bovine serum
GFP	Green fluorescent protein
HA	Hemagglutinin
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immnunoglobulin G

IL	Interleukin
ISCOM	Immune stimulating complex
Kb	Kilobases
KY	Kentucky
LB	Luria-Bertani
M	Matrix protein (Of influenza virus)
MDCK	Madin-Darby Canine kidney
MHC	Major Histocompatibility complex
NA	Neuraminidase
NBT	Nitro-blue Tetrazolium
NIH	National institute of health
NP	Nucleoprotein
NS	Nonstructural protein
OIE	Office Des Internationale Epizootics
PA	Polymerase A
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PNPP	Para-Nitrophenyl phosphate
RBC	Red blood cell
RNA	Ribonucleic acid
RT-PCR	Reverse transriptase polymerase chain reaction
SDS	Sodium Dodecyl Sulfate
SV40	Simian virus 40

TBS	Transfer buffer saline
TEMED	Tetramethylethylenediamine
Th	Helper T cells
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

1.1 Purpose of the study

Equine influenza is a highly contagious disease that has a significant impact on the horse industry (Kemen, Frank, and Babish, 1985; Kita, 1993; Powell *et al.*, 1995). The influenza virus is transmitted by aerosol during transportation of horses, and under conditions of overcrowding and stress. Infected horses develop respiratory symptoms that can culminate in secondary bacterial pneumonia. The resulting exercise intolerance leads to poor performance, and cancellation of race meetings, hence loss of revenue. The influenza virus has a very high frequency of mutation (Parvin *et al.*, 1986). It is the basis of antigenic drift (Gething *et al.*, 1980; Palese *et al.*, 1980) whereby the structure of the immunogenic, viral surface protein, the hemagglutinin (HA), changes frequently. Therefore, animals that have been previously vaccinated may not be protected from newly evolved strains.

Most of the current available vaccines rely on inactivated viruses. These vaccines are poorly immunogenic. Furthermore, a different antigen presentation pathway for the antigen is involved after intramuscular (i.m.) inoculation of inactivated vaccine when compared to a natural infection, and a significantly lower immunity is elicited. Hence the level of immunity is insufficient to protect horses from infection. One adverse consequence is that a vaccinated animal can be infected sub-clinically, without any overt symptoms. However, these animals can shed virus to infect others (Lai *et al.*, 1994). Furthermore, inactivated vaccines elicit only IgG responses, whereas IgA antibodies are

important in neutralizing the infecting virus at the mucosal sites (Clements and Murphy, 1986). The duration of immunity elicited by current inactivated vaccine is short. The development and testing of such vaccines is also a time consuming process. Sometimes, the demand for the vaccine cannot be met (Pound, 1980). Moreover, the strains that are incorporated may no longer be relevant to those in circulation, due to the long lag time for approval. Hence, there is an urgent need to develop a better alternative vaccine for this disease.

1.2 Hypothesis

It has been established that i.m. vaccination of inactivated influenza virus produces humoral immunity but no significant mucosal immunity (Clements and Murphy, 1986). However, intranasal vaccination of inactivated vaccine has been shown to produce significant elevation of serum IgA titers (Glueck, 2001). Similarly, live attenuated vaccines have been found to elicit a longer duration of immunity than that obtained by the use of an inactivated vaccine. Inactivated vaccines have the disadvantages of not targeting the required kind of immune response. Live, attenuated virus elicits mucosal immunity. However, in addition to the risk of reversion to the wild type, these vaccines harbor a potential danger of genetic reassortment with other viruses, creating new pathogenic strains.

A DNA vaccine is consisted of a plasmid encoding an immunogenic gene. The plasmid is introduced into host cells. The encoded protein is transcribed and translated in

the host cells. An immune response that resembles a natural infection is elicited (Gurunathan, Klinman, and Seder, 2000).

The HA protein is most important in eliciting protective immunity in influenza infections (Virelizier, 1975), as anti-HA antibodies neutralize the virus and inhibit the binding of the virus to host cell receptor. We hypothesized that by intranasal DNA vaccination with a plasmid encoding the HA gene, a protective mucosal immunity will be elicited. The HA protein would be synthesized *de novo* and will be expressed in its native form in the host. The antigen presentation pathway of the HA resembles to that of a natural infection. Stimulation of humoral immunity involving production of HA-specific IgG, long-term anamnestic responses, and the highly desirable mucosal response involving HA-specific IgA antibodies, will be elicited.

1.3 Overview of the research methodology

An expression vector pTOPO/V5/His was used to prepare the vaccine construct. The HA1 gene of equine-2 influenza virus, A/Eq/Kentucky/98, was inserted downstream of the eukaryotic pCMV promoter. The plasmid DNA was amplified in *Escherichia coli* cells.

To verify the expression of HA1, Madin-Darby canine kidney (MDCK) cells were transfected with the DNA construct, using lipofectamine or by electroporation. The cytosol and nuclear fractions were extracted, and subjected to a PCR to verify the efficacy of transfection. Total cell lysates were subjected to Western blot hybridization, using convalescent equine anti-KY/98 convalescent serum and goat anti-

A/Eq/Miami/1/63 typing antiserum. To evaluate the efficacy of the DNA vaccine, a vaccine trial using Balb/c mice was performed. Groups of four mice each were vaccinated intranasally and challenged with homologous A/Eq/KY/98 equine influenza virus. Anorexia is a major clinical symptom for mice infected with influenza virus. Significant difference in weight loss between vaccinated and unvaccinated groups was used to evaluate the degree of protection. Sera samples were also assayed for HA specific IgG and IgA antibodies.

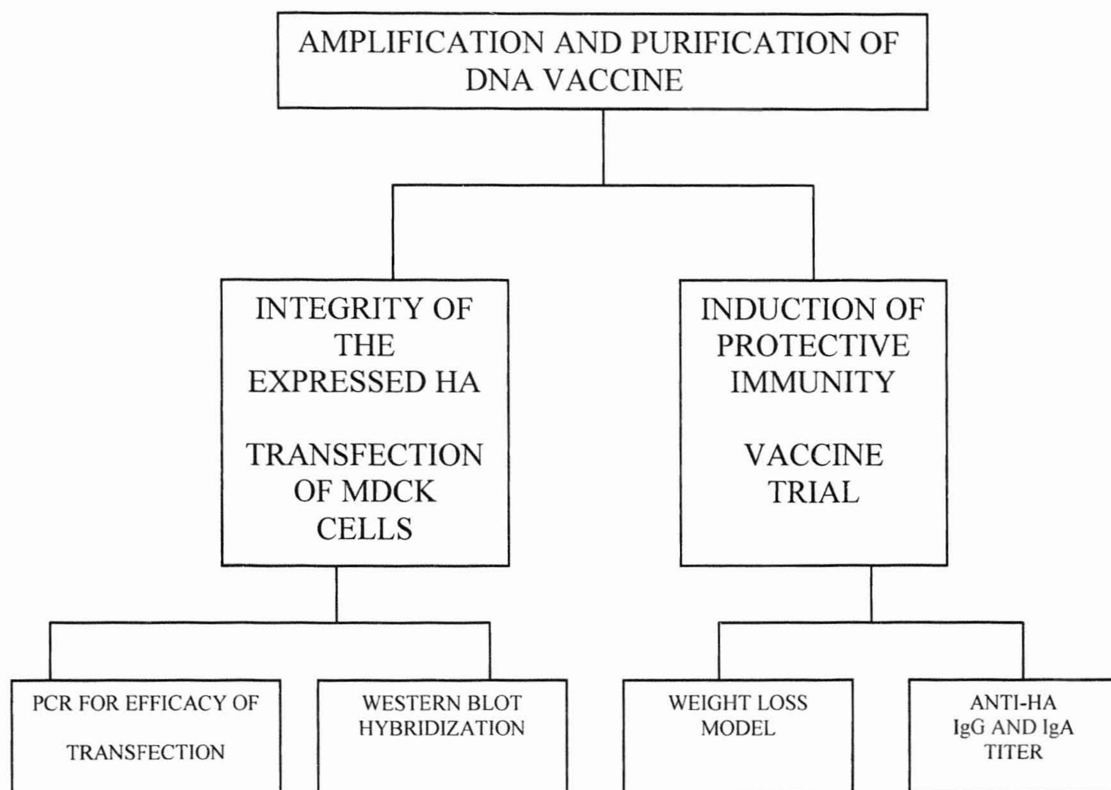

Figure 1-1: Research methodology: An overview of the research methodology described in this thesis.

1.4 Significance:

The discovery of DNA vaccines has exciting implications in the prevention of infectious diseases. This type of immunization is particularly useful for infections caused by intracellular parasites, and for those diseases where a particular type of immune response needs to be targeted. DNA vaccines have several advantages as a vaccine for influenza. Updating the vaccine, due to antigenic drift, in will be much simplified: it will involve only the cloning of the HA gene from the new strain, and the DNA vaccine vector can be used and considered as the original licensed vaccine. In contrast, manufacture of a conventional vaccine would involve adaptation of the strain to egg or cell culture, safety and efficacy testing.

DNA vaccine elicits immunity that resembles that of a live attenuated vaccine, and is stronger and longer lasting compared to the immunity elicited by an inactivated vaccine (Clements and Murphy, 1986). Problems associated with live vaccines, for example, reversion to virulence can be avoided. Furthermore, it has be shown that intranasal inoculation of antigen elicits mucosal IgA responses (Glueck, 2001). Mucosal immunity is highly desirable in the prevention of influenza infection. There have been no major side effects associated with the use of DNA vaccines (Makela, 2000). Plasmid DNA is a stable product that does not require sophisticated infrastructure for manufacture, transportation, delivery, and administered to the desired host.

CHAPTER II

LITERATURE REVIEW

2.1. Equine influenza

2.1.1 Classification of influenza viruses:

The *Orthomyxoviridae* family contains two genera: influenza A and B viruses; and influenza C virus. They are distinguishable based on the antigenic differences between the major internal proteins M and RNP. Influenza A viruses are further classified into subtypes based on the antigenicity of two surface viral proteins: the hemagglutinin (HA) and the neuraminidase (NA). There are 15 different HA and 9 different NA subtypes of influenza viruses identified so far. The nomenclature is according to the virus type, host, and place of origin; isolate number, and year of isolation. The equine viruses are influenza A viruses. The two major subtypes affecting horses are the H7N7 and H3N8. They are designated as equine-1 influenza and equine-2 influenza viruses, respectively.

Novel viruses of different subtypes can be generated when segments of the RNA genome from two genetically distinct virus strains can be randomly reassorted, leading to a major change in the antigenic structure of the virus, hence an antigenic shift (Gething *et al.*, 1980).

TABLE 2-1

CLASSIFICATION OF INFLUENZA A VIRUSES

Hemagglutinin	Humans	Swine	Horses	Birds
H1	PR/8/34	Sw/LA/15/30	None	Dk/Alb/35/76
H2	Sing/1/57	None	None	Dk/Ger/1215/73
H3	HK/1/68	Sw/Taiwan/70	**Eq/Miami/1/63**	Dk/Cz/56
H4	None	None	None	Dk/CZ/56
H5	HK/156/97	None	None	Tern/S.A./61
H6	None	None	None	Ty/Mass/3750/65
H7	None	None	**Eq/Prague/1/56**	FPV/Dutch/27
H8	None	None	None	Ty/Ont/6118/68
H9	HK/1/99	None	None	Ty/Wis/1/66
H10	None	None	None	Ck/Ger/N/49
H11	None	None	None	Dk/Eng/56
H12	None	None	None	Dk/Alb/60/76
H13	None	None	None	Gull/MD/704/77
H14	None	None	None	Dk/Gurjev/263/82
H15	None	None	None	Dk/Austral/341/83
Neuraminidase	**Humans**	**Swine**	**Horses**	**Birds**
N1	PR/8/34	Sw/la/15/30	None	Ck/Scot/59
	HK/156/97	Sw/Taiwan/70	None	None
N2	Sing/1/57	None	None	Ty/Mass/3750/65
	HK/1/68	None	None	None
	HK/1/99	None	None	None
N3	None	None	None	Tern/S.A/61
N4	None	None	None	Ty/Ont/6118/68
N5	None	None	None	Sh/Austral/1/72
N6	None	None	None	Dk/Cz/56
N7	None	None	**Eq/Prague/1/56**	FPV/Dutch/27
N8	None	None	**Eq/Miami/1/63**	Dk/Ukr/1/63
N9	None	None	None	Dk/Mem/546/74

Table 2-1: The two subtypes of equine viruses in circulation are the H3N8 and the H7N7. Most of the types are found in avian species. Waterfowl serves as reservoir of the virus. Pigs have been implicated as mixing vessels for reassortant viruses.

2.1.2 Structure of the influenza virus

The influenza virus is highly pleomorphic, can be spherical or filamentous in shape. The virus is enveloped and just beneath the membrane is the matrix protein (M), which is the most abundant protein. The viral genome is comprised of eight segments of negative-stranded RNA. Each segment encodes at least one protein. The two important surface proteins are the hemagglutinin (HA) and the neuraminidase (NA). They project as spikes from the viral surface. The function of HA is to initiate infection by binding to sialic acid receptors on susceptible host cells. The role of NA is to prevent the aggregation of viral particles, and in the release of progeny viruses. The nucleoprotein (NP) packages the eight single stranded RNA segments. Viral encoded RNA-dependent RNA Polymerases are PA, PB1 and PB2. Two nonstructural proteins comprise of NS1 and NS2. These proteins are associated with replication but are not found in progeny virions (Flint S.J, 2000).

2.1.2.1 Hemagglutinin structure and importance:

Hemagglutinin is the most important immunogenic protein of the virus. For quine-2 influenza virus, this viral RNA segment is consisted of 1762 nucleotides, and encodes a 565 amino-acid protein. The protein has two main domains, the HA1 and HA2. The monomer has a stalk that extends from the membrane and a globular head outside the membrane. The stalk is composed of an alpha-helix and includes portions of the HA1 and HA2 segments. The HA1 and HA2 segments are held by a disulphide bond that is

Australia and New Zealand are free of the disease so far (Mumford J.A, 1991; Wilson, 1993).

Three American and one Eurasian lineages of the H3N8 virus have been identified based on changes in the HA sequence. Both American and Eurasian lineages are present in Europe and the United Kingdom. Only one strain of the virus belonging to the Eurasian lineage has been isolated in the Western Hemisphere (Lai *et al.*, 2001). The diverged evolution of equine-2 influenza virus complicates the choice of vaccine strains in vaccine development. Outbreaks of equine influenza continue to occur, despite regular and repeated vaccinations (Chambers.M., 1999)

2.1.4 Transmission

Transmission of the influenza virus is mainly by the aerosol route. Infected animals exhale the virus during the initial stages of the disease, when they have the characteristic hacking cough. The level of immunity of the contact animals will determine the extent of transmission, and the intensity of symptoms. Previously unexposed animals and unvaccinated animals exhibit the worst symptoms. Some vaccinated animals, though protected from clinical disease, they can shed virus and infect others (Lai *et al.*, 1994; Mumford J.A, 1991). Sub-clinical infection is the most significant problem for the spread of the virus.

Transportation of horses is an important factor in dissemination of the disease over distances, and sometimes across continents (Powell *et al.*, 1995). The stress of transportation, and the proximity of animals in close confinement, exacerbates the spread of the virus. Outbreaks occur throughout the year, but are more frequent during the winter

months and during equine events, particularly during sales in the spring and in the fall. Other stressful conditions like weaning, training or labor of drought horses can exacerbate the condition. Although aerosol transmission is the primary means of spread, contaminated water, personnel and transport vehicles have been implicated in disease transmission (Guthrie, Stevens, and Bosman, 1999).

2.1.5 The disease and symptoms:

The influenza virus spreads rapidly and suddenly. High fever and a dry hacking cough are the primary symptoms of influenza, followed by respiratory distress. In the event of viremia, signs of myocarditis and congestive heart failure may occur. The virus causes inflammation and edema of the respiratory epithelia. Secondary bacterial infection may follow, and leads to bronchopneumonia. Animals that are forced to exercise despite symptoms may experience exercise-induced pulmonary hemorrhages (Gross *et al.*, 1998). Though the mortality is not high, all susceptible animals are likely to be affected. The disease can affect horses of all ages, but occurs most frequently in two to three year old racehorses that have not been previously exposed or vaccinated (Wilson, 1993).

Influenza is a very important animal health and economic problem for the equine industry (Mayr, 1987). Racehorses have to be withdrawn from training and exercise for long periods of time until their respiratory epithelium heals during course of recovery. An animal with a severely damaged respiratory epithelium may have to be euthanized. In case of an outbreak, entire race meetings may have to be cancelled, leading to loss of revenue (Powell *et al.*, 1995).

2.2 Current vaccines:

The *Office des Epizootics* (OIE) recommendations state that both the Eurasian and American lineages of equine2 influenza viruses should be incorporated in the vaccine, while the use of equine-1 influenza virus could be discontinued (Chambers.M., 1999). However, currently commercial vaccines contain both equine-1 and equine-2 influenza viruses. The herd immunity level influences the success of the vaccination program (Peterhans, Zanoni, and Bertoni, 1999). Worldwide surveillance of the disease is indispensable to effective control and vaccine manufacture.

In the production of vaccine, equine influenza viruses are cultivated in embryonated egg or in cell culture. After chemical inactivation of the virus, adjuvants, like aluminum hydroxide, oil and saponin, are added to enhance immunity. Adjuvant stimulates local infiltration of lymphocytes to the site of injection, and slows the release of the antigen to increase the duration of antigen stimulation. Side effects to the vaccine may include localized swelling, pain or transient fever and rarely anaphylactic reactions. Egg proteins, antigen and adjuvant in the product (Belgrave and Allpress, 1986) may induce these adverse reactions.

Foals are vaccinated twice, with a gap of three to six weeks, followed by a booster in the sixth month and annual vaccinations thereafter. Animals at high risk are vaccinated every three to four months (Morley *et al.*, 1999).

Current standard for vaccine potency is by the single radial immunodiffusion (SRD) method (Wood *et al.*, 1983). The OIE, however, recommends that animal

challenge experiments should replace the serological criteria in licensing of new vaccines (Chambers.M., 1999).

Improved vaccines for equine influenza include ISCOM (immune stimulating complex) vaccines that include purified HA and NA antigens; adjuvanted with a glycoside to form a complex lattice. These vaccines produce better and longer immune responses, and were marketed in Scandinavia and Britain, but they are no longer available (Sundquist, Lovgren, and Morein, 1988; Wilson, 1993). The use of cold adapted, attenuated live virus delivered intranasally has been another practical and successful approach to the problem. The virus replicates in the upper respiratory tract only and does not infect the lower respiratory tract, where the temperature is higher (Chambers *et al.*, 2001; Lunn *et al.*, 2001; Townsend *et al.*, 2001). However, the potential danger of reversion to virulence remains. Reassortant virus vaccines are composed of viruses with HA and NA proteins from human strains and nonstructural proteins from avian strains were found to have low levels of pathogenicity, and induced protection against the human strain. These approaches are of little practical use because live vaccines can revert to virulence (Snyder *et al.*, 1986). Expression of surface proteins in vaccinia was another approach for an alternative vaccine (De *et al.*, 1988), however, the practicality of such vaccine is doubtful.

2.2.1 Difficulties in vaccine development:

The antigenic drift of influenza virus is the most important factor that hinders its control. (Kawaoka, Bean, and Webster, 1989; Oxburgh *et al.*, 1994). The divergent

evolution of the equine-2 influenza virus into three evolutionary lineages makes selection of vaccine strains more difficult (Lai *et al.*, 2001). Constant monitoring of the disease all over the world, identification of emerging strains and constant evaluation of vaccine strains in terms of protection afforded against circulating strains is indispensable. Inadequacy of existing vaccines in terms of the type and duration of immunity elicited (Nelson *et al.*, 1998), lack of updated vaccines, and the spreading of the virus by subclinically infected animals further complicate the issue of prevention of this disease.

2.3 Immune response to influenza:

2.3.1 General principles of immunity:

The immune response is a physiological reaction to infection, which helps to contain and to eliminate the infectious agent during infection. It also possesses a memory function. When the agent is encountered again, the response to it is accelerated, and more specific. This phenomenon is called anamnesis. It is the basis for protection against infectious diseases by vaccination.

Immunity can be divided broadly into the innate immunity and adaptive immunity. Innate immunity is non-specific, but is the first line of defense and helps to limit the infection. The various components of innate immunity include macrophages, natural killer cells, interferon and the complement system. The microbe causes inflammation at the site of entry leading to release of immune active mediators and influx of neutrophils and lymphocytes. Phagocytic cells engulf the agent. Macrophages and dendritic cells act

as antigen presenting cells that interact with the mechanisms of acquired immunity. Increased levels of interferon are observed. The classical or alternate complement pathways are activated, leading to the lysis of the infectious agent.

The adaptive immune response is mediated by T cells and B cells. These cell types interact to produce a highly specific response. Antigen presentation to T cells requires association with the MHC class I and II receptors. The two important classes of effector T cells are CD4+ and CD8+ T cells. CD8+ T cells associate with MHC class I receptors and have cytolytic activity. CD4+ T cells are associated with MHC class II receptors. After activation, they differentiate into Th1 (Helper T cells type1) and Th2 (Helper T cells type2). IL-12, IL-2, IFN-alpha and TNF-beta control the Th1 response. This response activates the CD8+ cytotoxic T cells. IL-1 activates Th2 cells to produce IL-4, IL-5, IL-6, IL-10 and IL-13, which in turn activate B cells to produce antibodies. B cells produce antibodies of various classes including IgM, IgG, IgA, IgE and IgE. These antibodies can directly neutralize the virus or help in innate immunity by triggering off the complement pathway or antibody dependent cytotoxic cells (ADCC) (Kuby, 1992). IgM appears early in the response. IgA is present at the mucosal surfaces. IgG appears later in the response but is responsible for specific neutralization of the pathogen. IgE is involved in hypersensitivity reactions.

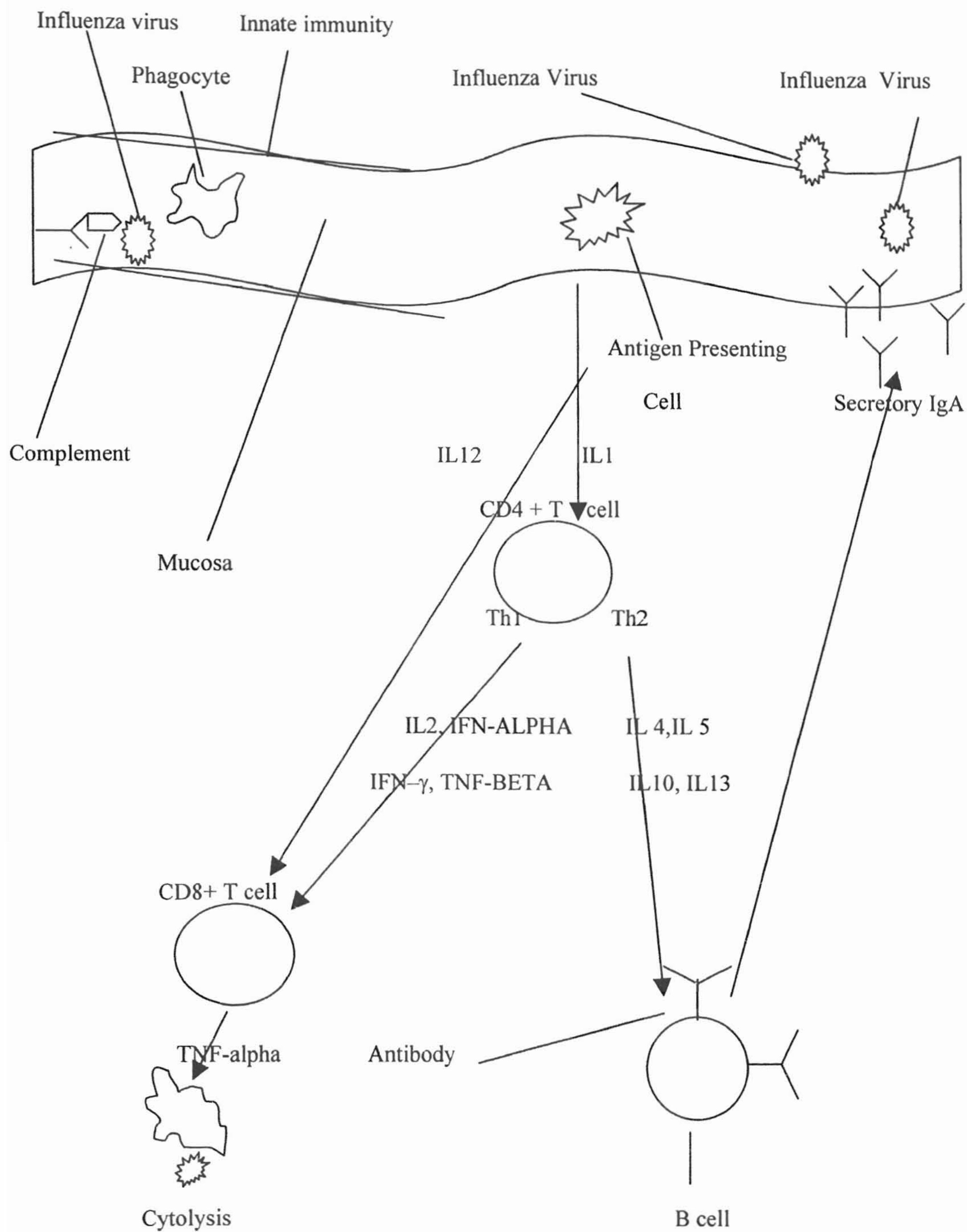

Fig 2-1: Immune response to influenza: Influenza virus gaining entry through the nasal mucosa, encounters both innate immunity and acquired immunity of the host. Production of secretory IgA is an important aspect of viral containment.

2.3.2 Role of innate immunity in influenza:

Innate immunity plays a role in limiting the initial insult by influenza virus infection. It limits dissemination of the virus and aids in the recovery. Natural killer cells play an important role early during the viral infection (Stein-Streilein, Guffee, and Fan, 1988). Macrophages and natural killer cells in the lungs phagocitize the virus and act as antigen presenting cells. They also produce alpha and beta interferon (Price, Gaszewska-Mastarlarz, and Moskophidis, 2000). The complement system is activated through the classical or alternate pathway. Activation of complement leads to the neutralization or lysis of the virus (Lambre *et al.*, 1983).

2.3.3 Role of cell mediated immunity in influenza:

T lymphocytes play a crucial role in the recovery from influenza infection. Macrophages and dendritic cells act as antigen presenting cells (APC). They interact with two types of helper T cells (Th): Th1 and Th2. On interaction with APC, Th1 cells secret IL-12, IFN alpha and beta, and TNF beta. These cytokines activates CD8+ T cells (cytotoxic T cells, CTL), and CD8+ cells recognize and cause the cytolysis of viral infected cells. Interaction of the antigen presenting cells with Th2 cells leads to the activation of CD4 positive T cells. CD4+ cells secrete IL-2, and other cytokines: IL-4, IL-5, IL6, IL-10 and IL-13. IL-2 signals the proliferation of other T cells, while IL-4, IL-5 and IL-6 stimulate B cell differentiation and antibody secretion.

MHC class II restricted CTL response in natural influenza infections is stronger than that elicited with inactivated vaccines. Therefore, mice that were immunized with live virus and later challenged with virulent virus were found to eliminate viruses in the lung better than mice vaccinated with inactivated virus (Kris *et al.*, 1985; Wells *et al.*, 1983). Cell-mediated immunity seems to confer some cross protection against heterologous challenge. This was evident when the heterotypic protection was abolished with the use of anti-thymocyte serum (Floc'h and Werner, 1978). However, CTLs can be responsible for pathological lung tissue damage, possibly a result of their cytotoxic activity (Yap, Braciale, and Ada, 1979). A systemic IgE response, also triggered by CTL, contributes to the symptoms of the disease.

2.3.4 Role of humoral immunity in influenza:

Specific antibodies are produced by plasma cells. Plasma cells are activated B cells. Antigen presenting cells stimulate Th2 cells to secrete cytokines that lead to the proliferation and differentiation of plasma cells. Initially, antibodies of IgM isotype are produced. On maturation, other isotypes are secreted, including IgG and IgA. IgA is a major component of nasal secretion, while IgG is more abundant in the lower respiratory tract (Gonchoroff *et al.*, 1982) and in serum. The duration of high antibody titer elicited by natural infections can last for over a year. The antibody titer is short lived elicited by inactivated vaccines (Glueck, 2001; Hannant, Mumford, and Jessett, 1988). This is attriibuted to a difference in the antigen presentation pathway. It has also been shown in mice that serum antibodies play a role in virus clearance, act in conjunction with T lymphocytes (Gerhard *et al.*, 1997). Antibodies also activate the classical complement

pathway, and can result in complement mediated lysis of infected cells. Antibodies may play a role in ADCC (antibody-dependent cytotoxicity) (Lambre *et al.*, 1983); however, the extent in virus clearance remains to be determined. HA specific antibodies appear to be important (Virelizier, 1975), as a lack of it results in incomplete protection. Antibodies to the matrix and nucleoprotein are also detected but they do not play a role in protection (Virelizier, Oxford, and Schild, 1976). The specificity of the response depends entirely upon the composition of the HA (Oxford *et al.*, 1979).

2.3.5 Importance of mucosal immunity against influenza infection:

Mucosal–associated lymphoid tissues (MALT) are located at mucosal surfaces like the gut, lungs, nose and others. T lymphocytes present in these areas secrete IL-4 in response to infection and stimulate B cells to produce IgA and IgE. These cells can also travel to other areas of the body (Davis, 2001).

Complete protection against natural influenza infections requires local IgA antibodies in the respiratory tract, as it is the site of initial virus attachment. Intranasal administration of IgA in influenza-infected mice has been found to confer protection (Renegar and Small, 1991b). When the IgA response was blocked, it was found that previously infected mice could become susceptible to re-infection with the same virus again (Renegar and Small, 1991a). Spread of the virus was arrested by formation of complexes with secretory IgA in the nasal lamina–propria (Kaetzel *et al.*, 1991). Previously exposed animals are able to mount a quicker secretory IgA response (Hannant *et al.*, 1989). Secretory IgG responses have also been implicated in this response

(Mbawuike *et al.*, 1999). Therefore, design of an effective vaccine for influenza should aim at stimulating both mucosal and systemic responses.

Intranasal delivery of live, inactivated, subunit and DNA influenza vaccines by several means like ISCOMS, virosomes, proteosomes, liposomes and polymers have demonstrated the ability to induce protective mucosal immunity (Gluck, 1999; Gruber *et al.*, 1993; Hu, Lovgren-Bengtsson, and Morein, 2001; Kadowaki *et al.*, 2000; Klavinskis *et al.*, 1997; Levi *et al.*, 1995; Payne *et al.*, 1995; Singh, Briones, and O'Hagan, 2001). The advantages of this method of delivery over other methods are that the vaccine is easy to deliver and does not require needles and syringes that are potential sources of disease transmission. Further, the nose is easily accessible, highly vascular, and has a large surface for absorption. This route elicits both systemic and mucosal responses. Distant mucosal cells can also be involved in the response due to dissemination of effector cells (Davis, 2001). It is a very cost-effective delivery system (Luce *et al.*, 2001). Furthermore, intranasal delivery of an inactivated influenza vaccine has been shown to induce B cell dependent heterotypic cross-protection (Tumpey *et al.*, 2001).

2.4 DNA vaccines:

When DNA expression vectors encoding genes for chloramphenicol acetyl transferase and luciferase were injected into muscle tissue, it was found that the encoded proteins were expressed by the transfected cells (Wolff *et al.*, 1990). Further study proved that plasmid DNA persisted in the cells for at least nineteen months but did not replicate in them. The plasmid DNA remains extra-chromosomal (Wolff *et al.*, 1992) though

inside the nucleus. This discovery had important implications in the field of vaccinology. DNA vaccine studies with plasmid DNA encoding immunogenic genes of various pathogens using several routes and means of delivery and in different animal models have been carried out since then (Klavinskis *et al.*, 1997; Montgomery *et al.*, 1993; Raz *et al.*, 1994; Sasaki *et al.*, 1998). It is found that this approach is particularly useful in eliminating infections by intracellular parasites, because both cell-mediated and humoral immunity are stimulated. DNA vaccines also have applications in cancer and allergy therapy (Cohen, 2000; Sato and Kasukawa, 2000). Therefore, DNA immunization can offset several of the problems associated with producing an effective vaccine for influenza.

2.4.1 Basic requirements of a DNA vaccine:

The plasmid DNA vaccine should contain a cloning site where the gene of interest can be incorporated. It should also contain an antibiotic resistance gene to enable selection. A bacterial origin of replication is required for replication in bacteria. Since the vaccine is used in mammals, a powerful promoter for enhanced expression in mammalian cells is useful (Robinson, Hunt, and Webster, 1993). Viral promoters from cytomegalovirus and SV40 virus are normally incorporated. Since plasmids are of bacterial origin, certain palindrome motifs of unmethylated cytidine-phosphate-guanosine dinucleotides (CpG) are known to stimulate natural killer cells to secrete interferon gamma and other cytokines that enhance the B cell immune response (Klinman *et al.*, 1996; Krieg *et al.*, 1995). Such CpG motifs are incorporated in most DNA vaccine

vectors. Polyadenylation sequences derived from SV40 virus or from bovine growth hormone are incorporated to stabilize mRNA transcripts.

2.4.2 Route of administration:

Transfection of mammalian cells with plasmid DNA encoding viral antigens has been known to lead to antigen expression in several cell types and stimulation of various antigen presentation pathways. Detectable levels of antigen or plasmid are not required for persistence of the response (Akbari *et al.*, 1999; Wolff *et al.*, 1992). The reason for this anomaly may be persistence of antigen in follicular dendritic cells or stimulation of an antigen independent response (Gurunathan, Klinman, and Seder, 2000).

In DNA vaccination, the route of administration has a direct correlation with the type of immune reaction that is elicited and the types of cells that are transfected. Upon gene gun vaccination, dendritic cells are directly transfected and they express the protein and present it to T cells (Akbari *et al.*, 1999; Porgador *et al.*, 1998). Intramuscular injections of plasmid DNA transfect muscle cells (Ulmer *et al.*, 1996). However, this route generates other T cell responses by migration of the plasmid DNA from the site of injection to lymph nodes where dendritic cells are transfected (Akbari *et al.*, 1999; Wolff *et al.*, 1990). Intradermal vaccinations lead to transfection of keratinocytes and Langerhans cells (Hengge *et al.*, 1998). The keratinocytes remain at the site of vaccination to stimulate a local response. Therefore, cross priming of cells is an important mechanism by which a response is elicited to DNA vaccines. Antigens expressed by transfected somatic cells may be taken up other professional antigen

presenting cells to stimulate T cell responses (Ulmer J.B, 1998). Mucosal administration of DNA by various routes like intranasally (Wong *et al.*, 2001), intra-tracheally (Fynan *et al.*, 1993) and in the genital tract (Livingston *et al.*, 1998), by aerosols (Stribling *et al.*, 1992) combined with adjuvant like cholera toxin (Ban *et al.*, 1997), liposomes (Klavinskis *et al.*, 1997), cytokine genes (Larsen *et al.*, 1998) and saponin (Sasaki *et al.*, 1998) have all demonstrated a consistent elevation in mucosal IgA levels.

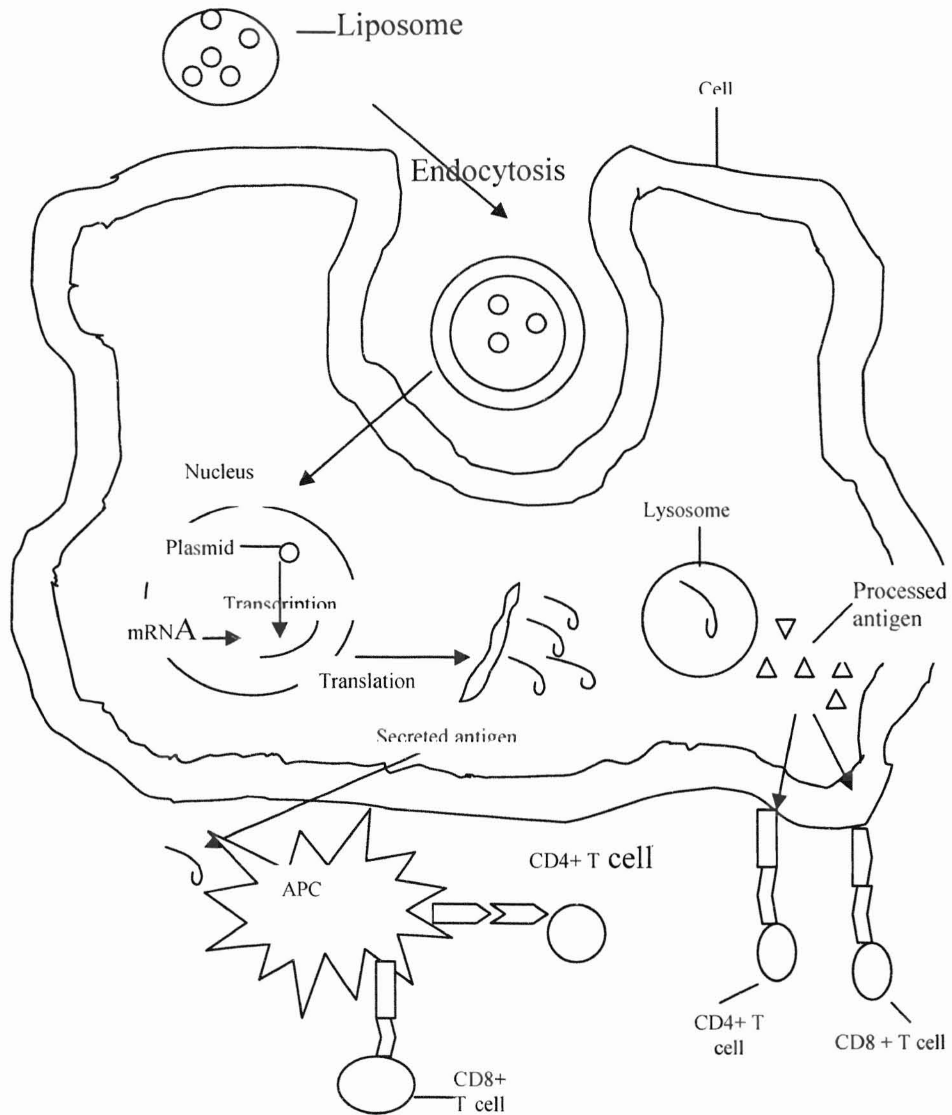

Figure 2-2: The mechanism of action of DNA vaccines: Fusion of the liposome with the cell membrane releases plasmid DNA into the cytosol. The antigen is transcribed, translated, secreted, and processed by antigen presenting cells. Cytosolic protein is engulfed by lysosomes, processed and presented in association with MHC class I molecules.

2.4.3 Immune responses to DNA vaccination:

2.4.3.1 Effects on cytotoxic T lymphocytes:

The major difference between immunity produced by a live viral infection and an inactivated vaccine is that the strength of the response is greater and the duration of immunity produced is much longer in a live viral infection. This can be attributed to the stimulation of CD8+ CTL (Kris *et al.*, 1985). The most important advantage of a DNA vaccine lies in the fact that it functions in a manner similar to that of an attenuated, live, vaccine. The antigen is synthesized endogenously. Therefore, a strong CTL response, which is specific and has a strong memory, is generated (Chen, Webster, and Woodland, 1998; Fu *et al.*, 1997).

2.4.3.2 Effects on Helper T cells:

Th1 and Th2 cells are functionally different products of the activation of CD4+ T cells. They are differentiated depending on the cytokines they secrete (Mosmann and Coffman, 1989). Th1 and Th2 responses are mutually suppressive (Mosmann, 1991; Parish, 1972). Intracellular parasites and viral infections require a Th1 response while extracellular; bacterial infections require a Th2 response.

A major advantage of the DNA vaccine is that the response can be modulated to suit the type of disease that it is targeted. This can be done by the incorporation of desired cytokine genes in the vaccine (Larsen *et al.*, 1998; Min *et al.*, 2001). Alternatively, by

including a secretory signal in the encoded protein can bias the response towards a Th2 response, while a protein localized in the cytoplasm will stimulate a Th1 response (Haddad *et al.*, 1997). Gene gun inoculations favor Th2 types of responses, and intramuscular injections stimulate a Th1 type response (Pertmer, Roberts, and Haynes, 1996). Intranasal administration favors a mucosal Th2, IgA response (Durrani *et al.*, 1998; Lundholm *et al.*, 1999). Due to the dichotomy of the Th1 and Th2 responses, DNA vaccination can be used to suppress one response by stimulation of the other. This approach is particularly useful in treatment of allergies where a Th2 response causes secretion of IgE (Raz *et al.*, 1996).

2.4.3.3 Effects on humoral immunity:

While innate and CTL responses help to contain the disease, humoral responses are indispensable in viral clearance, resolution of the disease and generating specific memory responses. DNA vaccines produce antigen specific responses and the classes of antibodies detected include IgG, IgM and IgA (Deck *et al.*, 1997). The dose, route and interval between vaccinations influence the magnitude of the response. In mice, about 10 –100 ug of plasmid DNA delivered intramuscularly, and 0.1 – 1.0 ug delivered using a gene gun have been shown to elicit an immune response (Fynan *et al.*, 1993). Booster immunizations with a four-week interval have been shown to increase serological responses (Deck *et al.*, 1997), (Fuller *et al.*, 1997). The results vary with the model used and the organism involved.

Peak antibody responses occur between 4 – 12 weeks after DNA immunization. Persistence of antibody levels for up to 1.5 years after vaccination (Deck *et al.*, 1997) has been recorded. Although antibody titers generated by a live vaccine have been shown higher than that generated by a DNA vaccine, this does not affect the protection elicited by DNA vaccines (Deck *et al.*, 1997). Since the protein will be produced in its native form, the type of antibody response generated will resemble that of a live viral infection (Peet *et al.*, 1997). Gene-gun vaccination of secreted antigen induces IgG response, and the response resembles a natural infection (Pertmer, Roberts, and Haynes, 1996). Intranasal DNA vaccinations elicit a mucosal IgA response (Sasaki *et al.*, 1998).

2.4.4 Advantages of DNA vaccination:

In summary, DNA vaccination is one the most versatile techniques that holds great promise for the future in the control of infectious diseases. Several immunogenic antigens can be incorporated in the same DNA construct to form a multi-gene vaccine. For influenza, currently prevalent strains can be incorporated in a timely fashion to meet market demands. Both cellular and humoral immunity are elicited. The protection elicited by these vaccines is comparable to live attenuated vaccines. The immune response can be modulated by several means to suit a particular disease. No specialized infrastructure is required for the production of the vaccine. Plasmid DNA forms a stable product and does not require cold chain preservation. Improved drug delivery systems make it easy to administer and cost-effective under field conditions.

Some concerns about use of these vaccines include the possibility that the DNA may integrate into the host chromosome. The host's normal cytokine responses may be irreversibly altered and can lead to autoimmunity. Immune tolerance, rather than immunity might be induced (Gurunathan, Klinman, andSeder, 2000). While extensive studies have not been conducted on these aspects, there have been no recorded adverse effects (Makela, 2000), except for an incidence of malarial DNA vaccine inducing immune tolerance rather than immunity in newborn mice (Ichino *et al.*, 1999). Significantly, the plasmid DNA does not integrate into the host chromosomes (Wolff *et al.*, 1992).

2.4.5 DNA vaccines and equine influenza:

The influenza virus has been a model organism in the study of DNA vaccines. In 1993 it was found that a HA expressing plasmid could confer protection against influenza (Robinson, Hunt, and Webster, 1993). Use of the NA gene conferred heterologous protection (Ulmer *et al.*, 1993). The immune responses involved were later characterized. Both cell mediated and humoral responses were found to be involved (Yankauckas *et al.*, 1993). Inclusion of both the HA and NA genes elicited both homologous and heterologous protection (Donnelly *et al.*, 1995; Montgomery *et al.*, 1993). Various routes of inoculation, including the gene-gun, mucosal and parenteral were compared (Fynan *et al.*, 1993). Various animal models, including ferrets (Donnelly *et al.*, 1997), baboons (Bot *et al.*, 1999) and mice (Justewicz and Webster, 1996) were used. The role of cytotoxic T cells (Fu *et al.*, 1997; Iwasaki *et al.*, 1997) and effects of

cytokine encoding genes (Larsen *et al.*, 1998; Lee *et al.*, 1999) in DNA vaccination against influenza were studied.

Similar studies on DNA vaccines for equine influenza were also carried out. Particle mediated delivery of HA encoding plasmid at skin and mucosal sites (tongue, conjunctiva and third eyelid) in ponies was found to stimulate IgG responses but a poor IgA response (Lunn *et al.*, 1999). However, the intranasal route was not used in this study. Immunization with a human IL-6 encoding gene along with the HA gene by gene-gun delivery was found to enhance viral clearance responses (Larsen *et al.*, 1998). The use of a gene-gun is impractical under field conditions, and the equine IL-6 needs to be characterized. The use of a recombinant A/Eq/KY/1/81 HA protein was expressed in a baculovirus system. Intranasal inoculation of this protein was compared with gene-gun vaccination of a HA encoding DNA vaccine. The DNA vaccine induced complete protection (Olsen *et al.*, 1997). Intranasal, liposome-mediated delivery of A/Eq/Pr/1/56 HA has been found to confer protection, and elicited both serum IgG and IgA antibody production (Wong *et al.*, 2001). The H3N8 subtypes of the virus used in this study are more relevant, as candidate vaccine strains since the H7N7 subtypes have not been recently recorded. This study is to test the use of only the HA1 to elicit protection, in contrast, most other studies have been using the complete HA sequence.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and methods:

The HA1 gene of equine-2 influenza virus, A/Eq/KY/98, was extracted from an allantoic fluid culture of the virus. The gene was cloned into a mammalian expression vector pTOPO 3.1 (Invitrogen, Carlsbad, CA). One clone was prepared without a 3' stop codon, and it's ORF was in frame with the C terminal His tag (designated pHA1). The second clone contains a 3' stop codon (designated pHA2). Competent *E. coli* cells were transformed with the plasmid. Clones were selected for the presence of ampicillin resistance. These clones were constructed and characterized by Lai et al (unpublished results). As described previously, these clones were further characterized *in vitro and in vivo*, for use as a DNA vaccine for equine influenza virus..

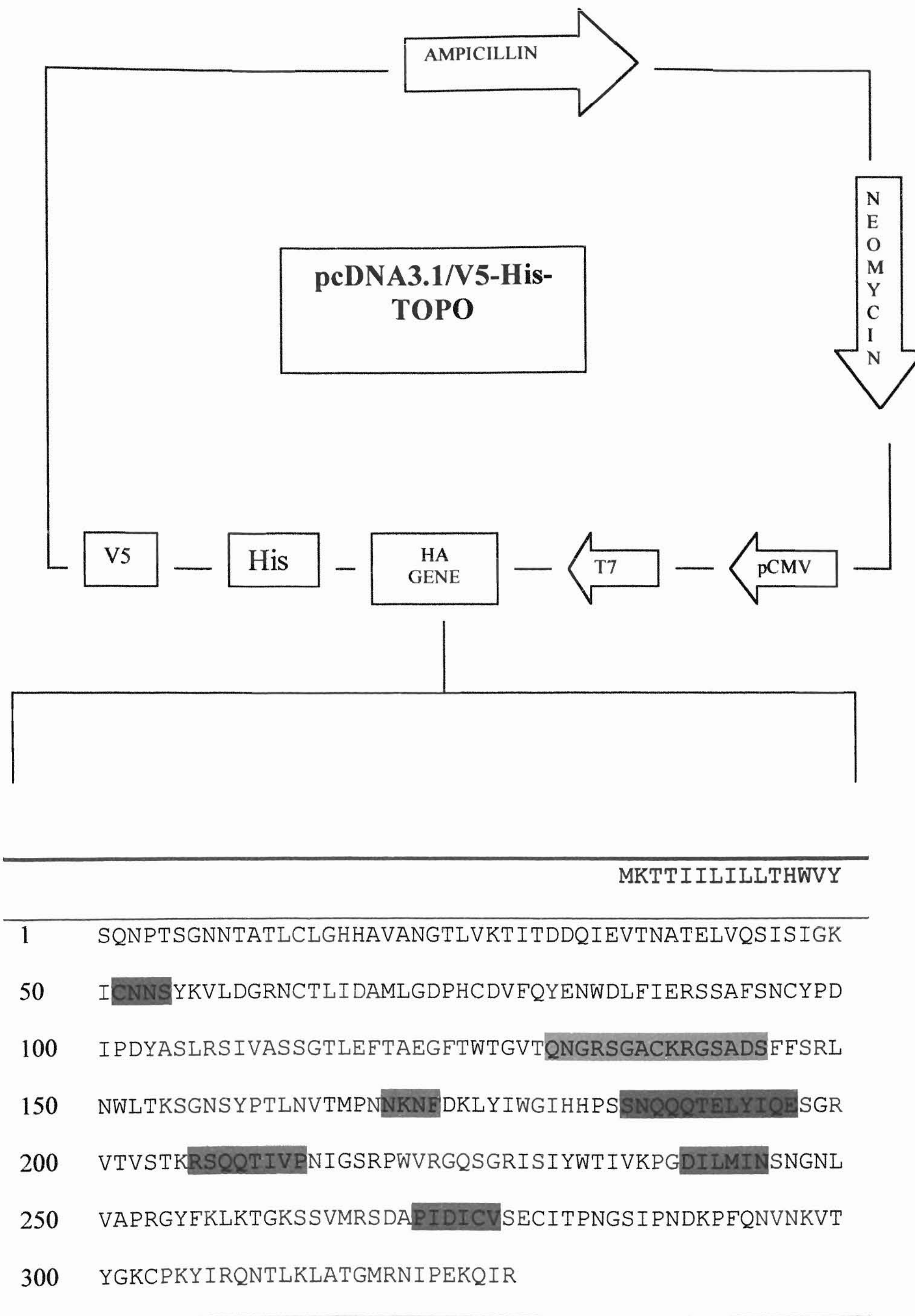

Fig 3-1: Map of the DNA vaccine construct: showing the pCMV promoter (5' of the HA gene), the His tag, and ampicillin resistance gene. The amino acid sequence the HA1 gene, including the signal peptide (shaded yellow), and the antigenic sites A, B, C and D (shaded Blue, Green, Pink and Red, respectively) are shown in the table below. Adapted from (Lai *et al*., 2001)

3.1.1 Restriction digest:

Plasmid DNA was extracted from the individual clones and subjected to restriction digestion for verification of the presence of the insert. Five micro liters of the extracted DNA were added to 0.5 ul of Hind III, 0.5ul of EcoRV, 1.0 ul of reaction buffer and 3.0 ul of distilled water to make a total reaction volume of 10.0 ul. The mixture was incubated for two hours at 37°C.

To visualize the DNA, 5.0 ul of the digested DNA was mixed with 5.0 ul of tracking dye (.25%(w/v) bromophenol blue, 20% glycerol, 0.1 M EDTA, pH 8) and loaded on a 1.5% (W/V) agarose gel. The gel was run for 20 minutes at 100 V in TBE buffer (89.0 mM boric acid, 89.0 mM Tris, 2.0 mM EDTA, pH 8). Ethidium bromide was added at 1.0 ug/ml concentration to stain the gel for 10 minutes. The gel was visualized under ultraviolet light, and the presence of the expected 1.0 Kb fragment was confirmed.

3.1.2 PCR for verification of the orientation of the insert:

Aliquots of lyophilized 2X MasterMIx PCR buffer (Promega, Madison, Wisconsin) were reconstituted to a volume of 17.0 ul with nuclease free water. The mix contains 50.0 mM Tris HCl (pH 9.0) and 3.0 mM MgC12, 400.0 uM each of dNTPs and 50.0 units/ml of Taq DNA polymerase in reaction buffer (pH 8.5). 1.0 ul of extracted plasmid DNA and 1.0 ul of T7 forward primer and 1.0 ul of reverse primer EH3-415+ were added to the tube to make up a total volume of 20.0 ul. The cycle parameters included denaturing at 95°C for 30 seconds, annealing at 42 °C for 30 seconds, extension at 72 °C for one minute

for a total of 30 cycles. A PCR thermocycler (Perkin-Elmer, Foster City, California) was used for the procedure. The presence of the expected 415 bp DNA fragment was confirmed by agarose gel electrophoresis.

TABLE 3-1

PRIMER SEQUENCES

PRIMER	SEQUENCE
EH3 29+	ATGAAGACAACCATTATTTG
EH3 1061-STOP	TCATCTGATTTGCTTTTCTGGTAT
EH3 -1061	TCATCTGATTTGCTTTTCTGG
T7+	TAATACGACTCACTATAGGG
EH3 415+	AACAAAATCTGGAAACTCT

Table 3-1:The nucleotide sequences of primers used for PCR of the HA gene and DNA vaccine construct are detailed here.

3.1.3 Transfection:

MDCK cells (ATCC, Rockville, MD) were used for transfection and in-vitro protein expression. Dulbecco's modified Eagle's medium (DMEM) containing ampicillin, streptomycin and amphotericin (Cellgro, Herndon, Virginia) were used for cell culture. Standard tissue culture methods as described by George V.G. et al. (George

V.G, 1996) were followed with modifications. Cells were incubated at 5% CO_2 tension in a CO_2 incubator (Revco, Asheville, NC).

3.1.3.1 Transfection by lipofectin:

Seventy to eighty percent confluent MDCK cells were harvested using trypsin EDTA. Cells were resuspended to 2 - 5 X 10^5 cells/ml. The suspension was used to seed a six-well tissue culture plate (Becton Dickinson, Lincoln Park, New Jersey). The plate was incubated overnight in the CO_2 incubator at 5% CO_2 tension.

Three tubes, each containing 200.0 ul serum-free DMEM. Each tube was added either 2.0 ug of pHA1, pHA2, or pGFP DNA. Twenty-four micro liters of Lipofectin (1.0 mg/ml solution) (Gibco BRL, Grand Island, New York) was added to 600.0 ul of serum free DMEM in a separate tube, and 200.0 ul of this solution was added to each of the above DNA–DMEM mixtures. The DNA-liposome mixtures were incubated at room temperature for 30 min for the formation of DNA-liposome complexes. The solutions were layered onto a monolayer of MDCK cells in a 35 mm petri dish. Six hundred micro liters of serum-free medium was added to all the wells to a final volume of 1.0 ml. The plate was incubated for 5 hours in the CO_2 incubator, followed by an additional 1.0 ml DMEM containing 10% FBS. The plates were further incubated overnight.

After 24 hours, cell sheets from the wells were scraped off using a pre-sterilized rubber policeman. Cells were washed three times in PBS, and lysed by re-suspending in hypotonic saline solution for 10 minutes. The cells were centrifuged at 1000g for 1.0 minute. The supernatant was collected as the cytosol fraction. The pellet was resuspended

in sterile distilled and deionized water (ddH_2O), and centrifuged at 1000g for 1.0 minute. The supernatant was collected as the nuclear fraction.

PCR reactions were performed on both nuclear and cytosolic fractions to verify the presence of the DNA as a measure of transfection efficiency. Forward primer EH3-29+ and reverse primer EH3-1061- were used. The presence of 1.0 Kb DNA fragment in the nuclear and cytosol fractions indicates the presence of pHA1 or pHA2 into the transfected cells. Cells transfected with pGFP were used as negative control.

3.1.3.2 Transfection by electroporation:

Transfection of cells was carried out as per the procedure of Chu et al with modifications (Chu, Hayakawa, and Berg, 1987). Seventy to eighty percent confluent cells were harvested by washing the cell sheet with PBS, and incubating at 37° C for ten minutes after addition of 0.5 ml of Trypsin EDTA. The cells were collected into a sterile 5ml tube and washed with PBS three times by centrifugation at 500g for 1.0 minute. They were re-suspended to 2 -5 X 10^6 cells /ml in DMEM without serum. The suspension was distributed in 400.0 ul volumes in three eppendorf tubes. 20.0 ul of 0.5 ug/ul of purified plasmid DNA, from clones designated as pHA1 and pHA2, were added to the tubes and allowed to stand for 10 minutes at room temperature for mixing. The mixture was then loaded into 4.0 mm disposable electroporation cuvettes (BTX) and pulsed in an electroporator (BTX - ECM 39, Genetronics) at 260 V, 1050 uF, and for 29 microseconds. The cells were incubated in the CO_2 incubator for 36 hours. Untransfected MDCK cells were used as a negative control.

3.1.4: Western Blot hybridization:

3.1.4.1 Preparation of total cell lysates of transfected mammalian MDCK cells:

Transfected cell monolayers in tissue culture flasks were washed three times with PBS, followed by scrapping with a rubber policeman. The cells were collected into Eppendorf tubes. They were washed with 500.0 ul of PBS twice, and re-suspended in 50.0 ul of Cell lytic B buffer (Sigma, St. Louis, MO), followed by incubation at room temperature for five minutes. The cell lysates were centrifuged at 1000 g for 5 minutes. The supernatant was distributed in 25ul volumes in Eppendorf tubes, and preserved at –80 °C.

3.1.4.2 Polyacrylamide gel electrophoresis:

The procedure of Laemaeli et al (Laemmli, 1970) was followed with modifications. A 12% gel was cast using the miniprotean II (Biorad, Hercules, California) electrophoresis module. Samples were prepared by adding 25.0 ul of 2X sample buffer to 25.0 ul of sample, and boiling at 100 °C for 5 minutes. Thirty microliter of sample was added to each well. Medium range molecular weight markers (Sigma, St. Louis, MO) were added to well 1 and 2, followed by adding purified equine-2 influenza virus, A/Eq/KY/98, as a positive control. Another negative control was provided by untransfected MDCK cell lysate. The gel was run at 150 V for 45 minutes, using 1X running buffer (Biorad, Hercules, California) and a power pack (Biorad, Hercules,

California). Lane 1 was excised and stained with Coomasie blue and de-stained overnight to verify proper protein separation on the gel.

3.1.4.3 Protein transfer to nitrocellulose membrane:

Western blot transfer and staining conditions were carried out using the procedure described by Hartshorn et al. with modifications (Hartshorn *et al.*, 2000). The SDS-PAGE gel was equilibrated in transfer buffer for 45 minutes. The nitrocellulose membrane was cut to the correct dimensions and pre-wetted in transfer buffer, along with two fiber pads and filter paper cut to size for 45 minutes. The transfer cassette was arranged by placing the fiber pad, followed by filter paper, gel, nitrocellulose membrane, filter paper and a fiber pad. The cassette was placed in the Mini Trans-blot apparatus (Biorad, Hercules, California) following the color code for electrode orientation with respect to the cassette. Transfer was carried out at room temperature, at 300 mA and 70 V, for 3.5 hr. The presence of the pre-stained protein markers on the nitrocellulose membrane confirmed the transfer of protein. The gel was stained with Coomasie blue and de-stained overnight to verify transfer.

3.1.4.4 Western blot hybridization:

The nitrocellulose membrane was incubated overnight at 4°C in blocking buffer. It was washed three times for ten minutes in 1x TBS without methanol, and incubated for 2 hours with antibody. A 1:3000 dilution of equine convalescent anti-A/Eq/KY/98 serum,

and 1:3000 goat anti-A/Eq/Miami/1/63 serum in blocking buffer, was used on two membranes with transferred cell lysate samples. The membrane was washed again with TBS, three times and incubated with 1:3000 dilution of secondary antibody in blocking buffer for 2 hours. Either anti-equine or anti-goat IgG alkaline phosphatase-conjugated antibodies (Sigma, St. Louis, MO) were used for the respective membrane. The membrane was then washed three times for ten minutes in washing buffer containing 1.0 % Triton X 100 and 5mM EDTA. One tablet of BCIP/NBT (Sigma, St. Louis, MO) substrate was dissolved in 10.0 ml of distilled water. The membranes were incubated in this solution for 30 minutes. The reaction was ended with the stopping buffer. The membranes were photographed using an image analyzer (Alpha Innotech, San Leandro, CA)

3.1.5 Mouse challenge study:

3.1.5.1 Experimental design:

This study was adapted with modifications from methods followed by Olsen et al (Olsen *et al.*, 1997). Groups of four, 20 day-old female BALB/c mice, selected at random, were separated into the following groups: 1) PBS; 2) inactivated KY/98 virus; 3) Green Fluorescent Protein; 4) pHA1 for homologous challenge; 5) pHA1 for heterologous challenge; and 6) pHA2. The inactivated virus inoculated group served as the positive control, both the pGFP group and the PBS group were serving as the negative control groups. Of the two pHA1 groups, one group was used for homologous challenge

with the homologous virus, A/Eq/KY/98, while the other group was used for heterologous challenge, A/Eq/Prague /56 virus.

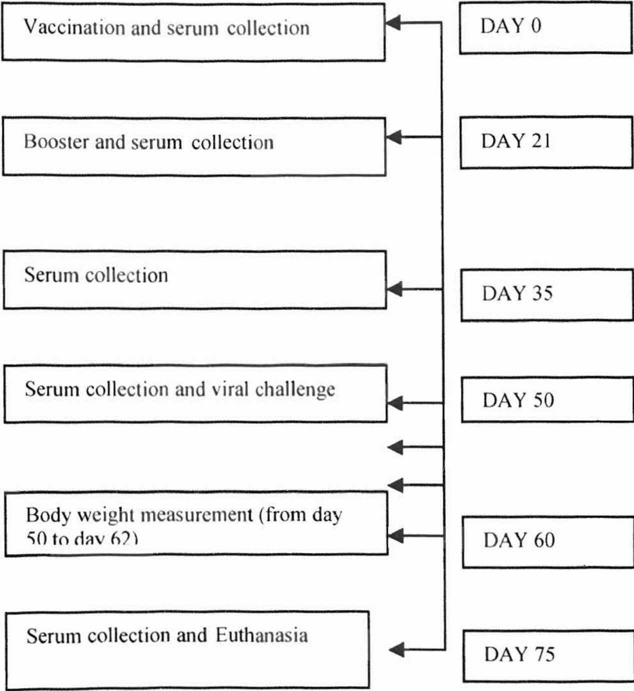

Fig 3-2: Schedule of the mice challenge experiment.

TABLE 3-2

MOUSE MODEL TO EVALUATE THE DNA VACCINE

Mice Group	TREATMENT	NO. OF BALB/C MICE	DOSE & ROUTE	SERUM COLLECTION (DAYS)	VIRAL CHALLENGE
1	PBS (NEGATIVE CONTROL)	4	20 UL	0, 21, 35, 50 & DAY 15 POST CHALLENGE	KY/98
2	KY 98 INACTIVATED VIRUS (POSITIVE CONTROL)	4	0.32 HA UNITS/G BODY - WEIGHT	0, 21, 35, 50 & DAY 15 POST CHALLENGE	KY/98
3	PHA1	4	0.01 UG DNA/G BODY - WEIGHT	0, 21, 35, 50 & DAY 15 POST CHALLENGE	KY/98
4	PHA1	4	0.01 UG DNA/G BODY - WEIGHT	0, 21, 35, 50 & DAY 15 POST CHALLENGE	PR/56
5	PHA2	4	0.01 UG DNA/G BODY - WEIGHT	0, 21, 35, 50 & DAY 15 POST CHALLENGE	KY/98
6	PGFP (NEGATIVE DNA CONTROL)	4	0.01 UG DNA/G BODY - WEIGHT	0, 21, 35, 50 & DAY 15 POST CHALLENGE	KY/98

Table 3-2: 6 groups containing 4 mice each were vaccinated with 0.01 ug DNA/g body weight intranasally, followed by a booster 3 weeks later. Mice were bled on 0, 21, 35, 50 days post inoculation, and 15Th days posts virus challenge. They were challenged on day 50. The body weight of each mouse was measured each day for 10 days after challenge with live virus.

3.1.5.2 Vaccine formulation:

A 20-ul dose/mouse containing 0.01ug DNA per gram body weight was formulated. The dosage contained 20.0 ug of Lipofectamine (GibcoBRL, Grand Island, New York). Lipofectamine is available as a 1.0-mg/ml solution. Purified plasmid DNA, pHA1, pHA2 and pGFP were prepared. The purified pHA1 DNA contained 0.1ug/ul, pHA2 contained 0.05ug/ul and plasmid pGFP contained 0.1 ug/ul as assessed by visualization on a 1.5% agarose gel. The required quantities of DNA and lipofectamine in DMEM were mixed and allowed to stand at room temperature for 20 minutes for the formation of complexes in accordance with the manufacturer instructions.

Twenty micro liters of PBS was administered intranasally to the PBS group. The KY98 inactivated vaccine group was administered 0.32 HA units/g body weight intranasally. The inactivated vaccine was prepared from an allantoic fluid culture of KY/98 virus with a HA titer of 1:16. The culture was inactivated by placing 1.0 ml of the virus suspension in a petridish, without lid, at a distance of 15 inches from a 30 W UV lamp, and irradiated for 15 minutes.

3.1.5.3 Anesthesia of mice:

All bleeding and vaccination procedures were carried out under Isofluorane (Forane, 1-chloro-2, 2,2-trifluoroethyldifluoromethyl ether) induced anesthesia as per the method of Wiersema with modifications (Wiersema *et al.*, 1997). A cotton pad soaked with 0.5 ml of 2% Isofluorane (Abbot labs, North Chicago, IL) was placed in a closed

glass jar. Mice were held in the jar until loss of pedal and ocular reflexes. An average time of one and a half minutes was required. Bleeding and vaccination were carried out when mice were completely anaesthetized.

3.1.5.4 Mice vaccination and bleeding protocol:

Mice were bled on day 0 and vaccinated intra-nasally with the primary dose of DNA vaccine and inactivated vaccine. They were bled and boosted with the same dose and by the same route on day 21. They were bled again on day 35 but were not given a booster vaccination. All groups of mice were bled again on day 50 and were challenged by intranasal inoculation of 50ul an allantoic fluid culture of A/Eq/Ky/98 virus with a HA titer 1:120 HA units. The heterologous challenge group was challenged with 50 ul of an allantoic fluid culture of A/Eq/Prague/56 culture with a 1:64 HA titer. Mice body weights were recorded before challenge and thereafter for 10 days as per the procedure followed by Solorzano et al (Solorzano *et al.*, 2000). All mice were bled on the 15th day post challenge and euthanized by Isofluorane® anesthesia, followed by cervical dislocation.

3.1.6 ELISA

Sera samples collected from the mouse challenge experiment were subjected to an ELISA for assessment of HA specific IgG and IgA antibodies. The method adopted from Deck et al (Deck *et al.*, 1997) was followed with modifications. 96 well ELISA plates (Nalge Nunc, Rochester, NY) were coated overnight with 1: 500 dilution of egg allantoic

fluid harvested KY98 virus in coating buffer at room temperature. The plates were washed three times with PBS containing 0.05%. Tween-20. They were blocked using blocking buffer containing 1.5% BSA for 2 hours. The plates were washed three times again and a 1:10 dilution of the serum in blocking buffer was added in triplicate. Normal mouse serum was used as a blank and inactivated virus inoculated mouse serum was used as a positive control. The plates were incubated on a plate shaker at room temperature for 1 hour. The plates were washed three times with washing buffer and a 1:3000 dilution of anti mouse IgG and IgA conjugate were added to the respective plates. After an hour's incubation at room temperature, the plates were washed and 1mg/ml of pNPP (Sigma, St. Louis, MO) in glycine buffer was added. The plates were read after 150 minutes at 405 nm using the ELISA plate reader (Biotek Instruments, Winooski, VE).

3.1.7 Statistical analysis:

Statistical analysis and graph construction was carried out using Microsoft Excel software. The standard error of the mean was obtained and represented graphically. A Student t test was performed on the challenge experiment and ELISA data. Levels of significance were determined using a 95% confidence interval.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Validation of the DNA vaccine construct:

The plasmid DNA construct required to be validated in terms of: a) The presence of the HA gene insert; and b) The orientation of the insert with respect to the plasmid DNA so that the 5' end of the gene is in frame with the CMV promoter.

4.1.1 Restriction digest:

Figs 4-1 and 4-2 are photographs of agarose gels with restriction digestion samples of plasmids pHA1 and pHA2. The 1.0 Kb band in the sample lanes 3, digested with two enzymes, indicates that the complete 1 Kb HA1 DNA fragment was correctly inserted into the vector.

M1 1 2 3

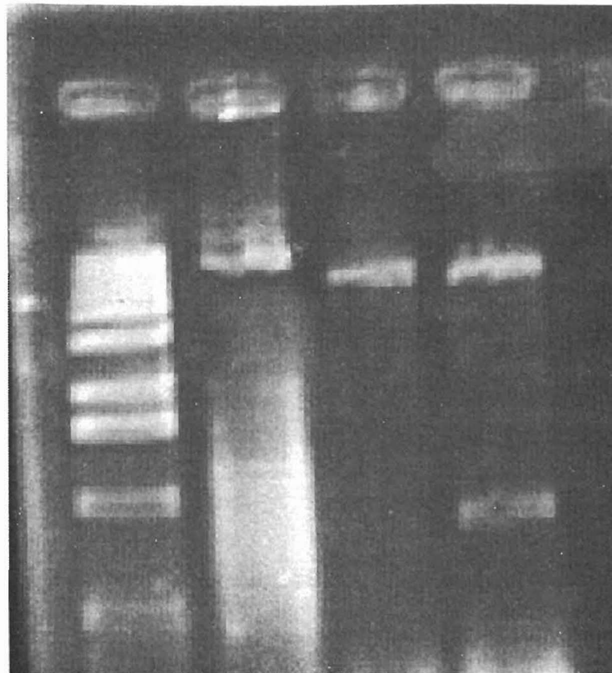

Fig 4-1: Restriction digestion of pHA1: Purified plasmid DNA was subjected to restriction digestion and electrophoresis in 1.5% agarose gel at 100 V for 30 minutes Lane M1: 1 Kb DNA ladder; Lane 1: Undigested plasmid DNA; Lane 2: DNA digested with EcoR V; Lane 3: DNA digested with EcoR V plus Hind III.

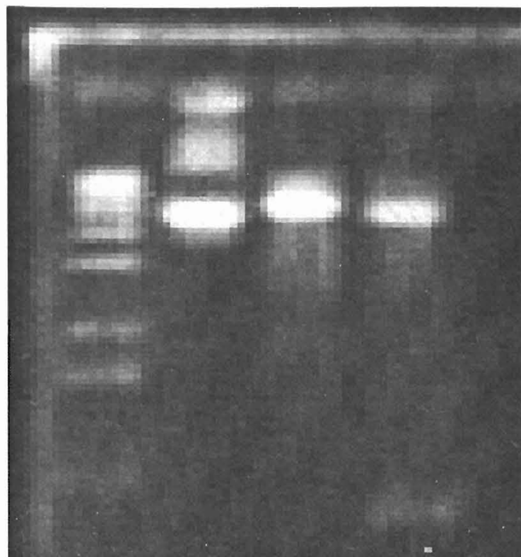

Fig 4-2: Restriction digestion of pHA2: Purified plasmid DNA was subjected to restriction digestion and electrophoresis in 1.5% agarose gel at 100 V for 30 minutes. Lane M1: 1 Kb DNA ladder; Lane 1: Undigested plasmid DNA; Lane 2: DNA digested with EcoR V; Lane 3: DNA digested with EcoR V and Hind III.

4.1.1 PCR for verification of the orientation of the insert:

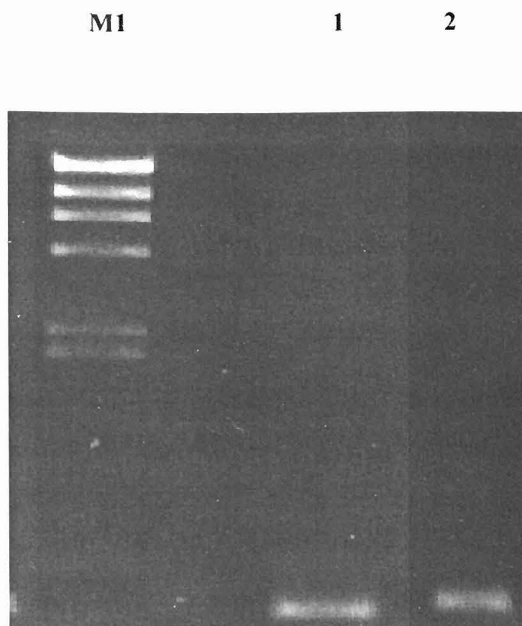

Fig 4-3: PCR of pHA1 and pHA2: Purified plasmid DNA pHA1 and pHA2 were subjected to PCR using a forward primer specific to the T7 promoter and a reverse primer specific to an internal site in the HA gene Lane M1: 1Kb ladder; Lane 1: pHA1; Lane 2: pHA2.

The orientation of the insert in terms of the 5' end of the HA gene being in frame with the CMV promoter on the plasmid and the 3' end of the gene being in frame with the tag was verified using PCR. A forward primer was specific to the 5' T7 promoter on the plasmid backbone and a reverse primer specific to an internal site 415 bps down stream of the HA gene were used. The expected 415-bp product is evident in lanes 2 &3.

4.2 Transfection:

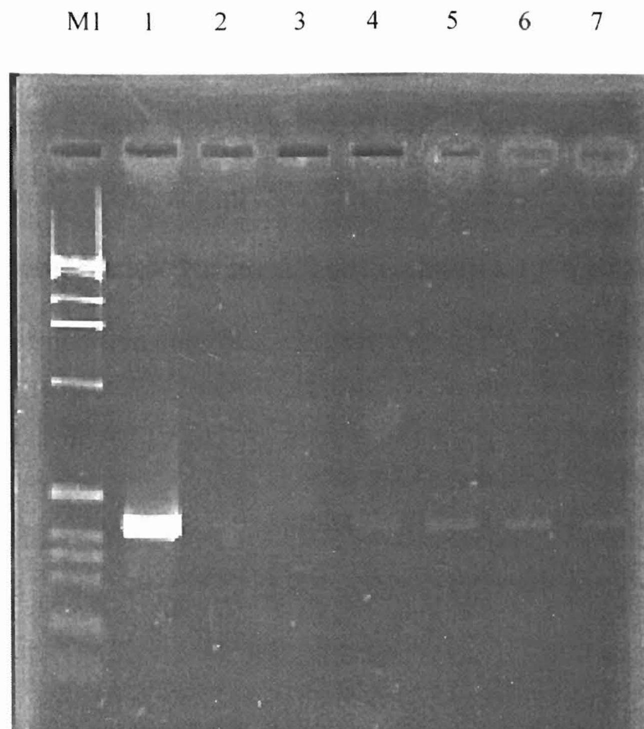

Figure 4-4: Transfection: Nuclear and cytosolic fractions from cells transfected with pHA1, pHA2, and pGFP were subjected to PCR with EH3-29+ as the forward primer and EH3-1061- as the reverse primer. Lane M1: 1 Kb DNA ladder; Lane 1: PCR + control; Lane 2: pGFP cytosol fraction; Lane 3: pGFP nuclear fraction; Lane 4: pHA1 cytosol fraction; Lane 5: pHA2 nuclear fraction; Lane 6: pHA2 cytosol fraction; Lane7: pHA2 nuclear fraction.

Cytosol and nuclear fractions from cells transfected with pHA1 and pHA2 were subjected to PCR to ascertain the presence of the plasmids within the cells upon successful transfection. Forward primer EH3 29 and reverse primer EH3 1061 were used. Plasmid pGFP was used as a negative DNA control. The expected 1.0 Kb band is evident in Lanes 4, 5, 6, and 7, which are cytosolic and nuclear fractions of cells transfected with plasmids pHA1 and pHA2. The bands are not evident in Lanes 1 and 2 that are pGFP cytosol and nuclear fractions. Lane L shows a 1.0 Kb DNA ladder and lane C the product of a PCR positive control.

4.3 Western blot:

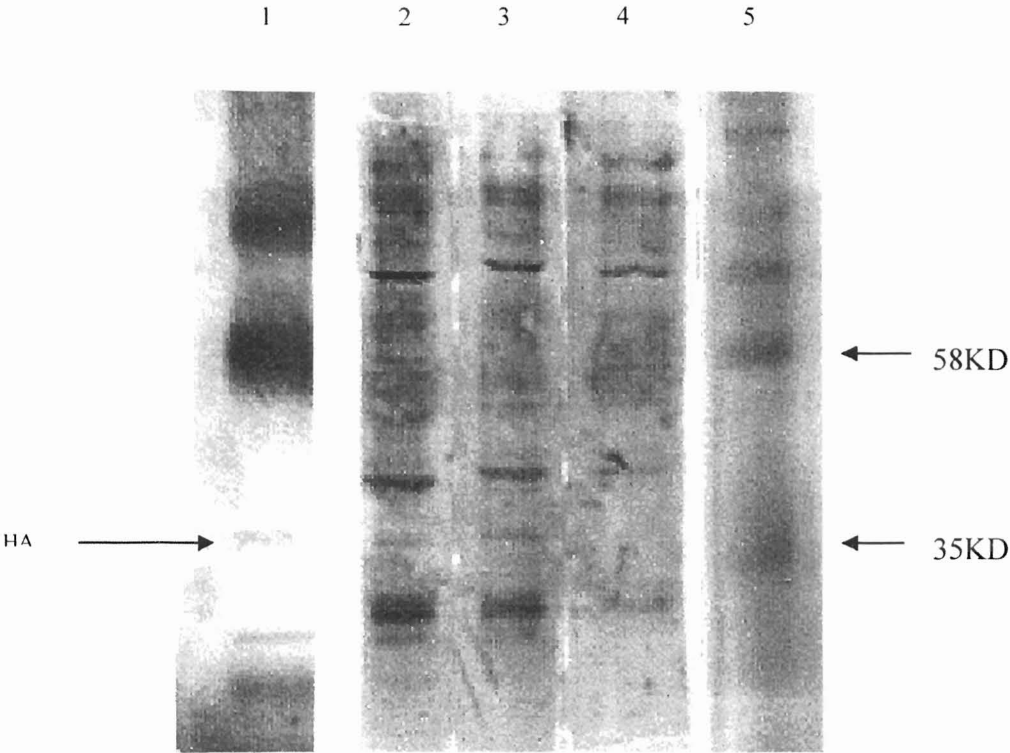

Fig 4-5: Western blot with equine anti-KY98 convalescent serum: Total cell lysates of MDCK cells transfected with plasmids pHA1 and pHA2 hybridized with equine anti-KY98 convalescent serum. Lane 1: A/Eq/Ky/98 virus; Lane 2: pHA1 total cell lysate; Lane 3: pHA2 total cell lysate; Lane 4: Untransfected MDCK cells; Lane 5: Molecular weight markers

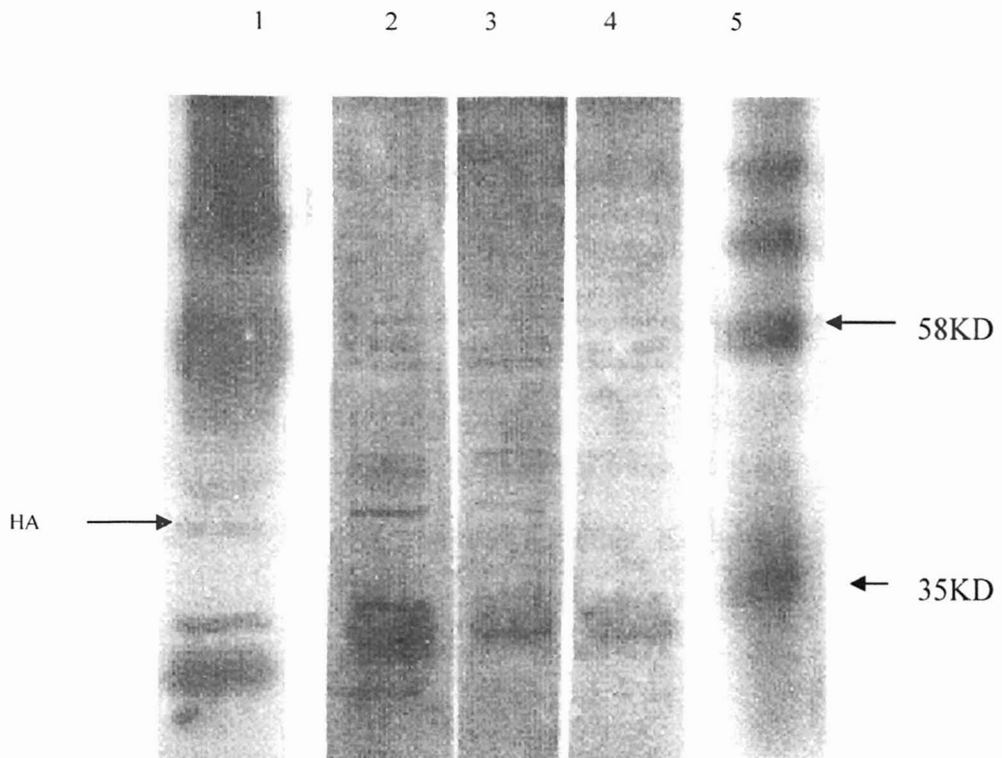

Fig 4-6: Western blot with goat anti –A/Eq/Miami/1/63 serum: Ttotal cell lysates of MDCK cells transfected with plasmids pHA1 and pHA2 hybridized with goat anti-A/Eq/Miami/1/63 antiserum. Lane 1: A/Eq/Ky/98 virus; Lane 2: pHA1 total cell lysate; Lane 3: pHA2 total cell lysate; Lane 4: Untransfected MDCK cells; Lane 5: Molecular Weight markers

To confirm the presence of antigen in vitro, total cell lysates from mammalian MDCK cells, transfected with plasmids pHA1 and pHA2, were subjected to Western blot hybridization. Untransfected MDCK cells were used as the negative control and A/Eq/KY/98 virus was used as a positive control. Since a 1.0 Kb DNA fragment encoding the HA1 segment of the HA gene was cloned, and expressed in mammalian cells, the product would have a native glycosylation pattern including about 20% of the molecular weight as carbohydrates (Gray and Tamm, 1998; Sugiura *et al.*, 2001). An approximately 40 KD band was expected to be present in the lanes with transfected cell samples (Arora *et al.*, 1985) and absent in the untransfected cell control. Two replicates of blotted membranes with transferred proteins were probed with two types of antiserum, convalescent, equine, anti-KY/98 serum and goat anti-A/Eq/Miami/1/63 antiserum. An approximately 40 KD band is present in the lanes for transfected cells and is absent in the untransfected control. The presence of several other bands is due to the fact that polyclonal serum was used.

4.4 Mouse protection study:

4.4.1 Post - challenge body weight loss:

Post-challenge body-weight loss and recovery were taken as the criteria (Solorzano *et al.*, 2000) to assess the severity of symptoms and protection conferred by the DNA vaccine. The mean weight loss in treatment groups is compared with that of the unvaccinated group in the graphs in Figs 4-7A to 4-7E.

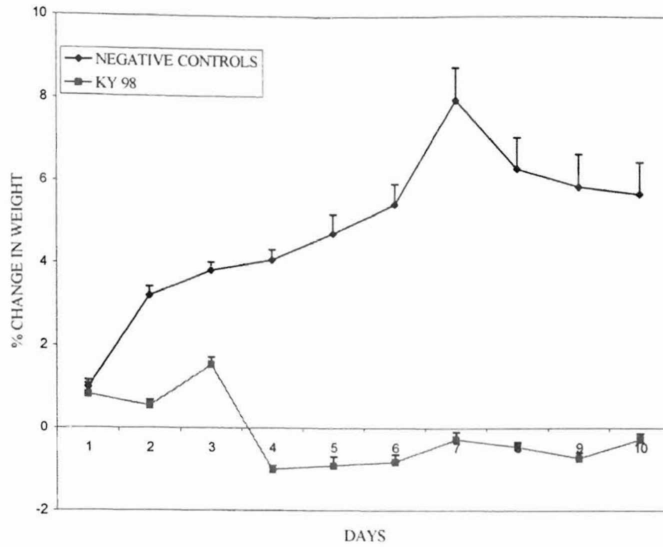

Figure 4 –7A: Post – challenge body weight loss – inactivated vaccine.

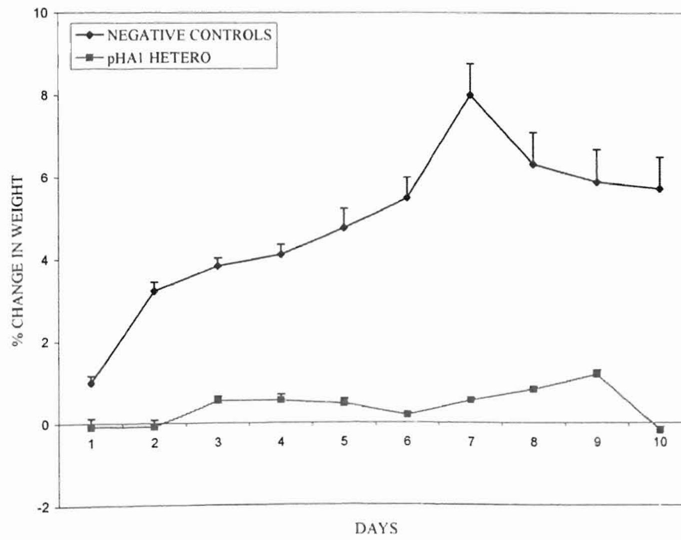

Figure 4 – 7B: Post – challenge body weight loss – heterologous challenge.

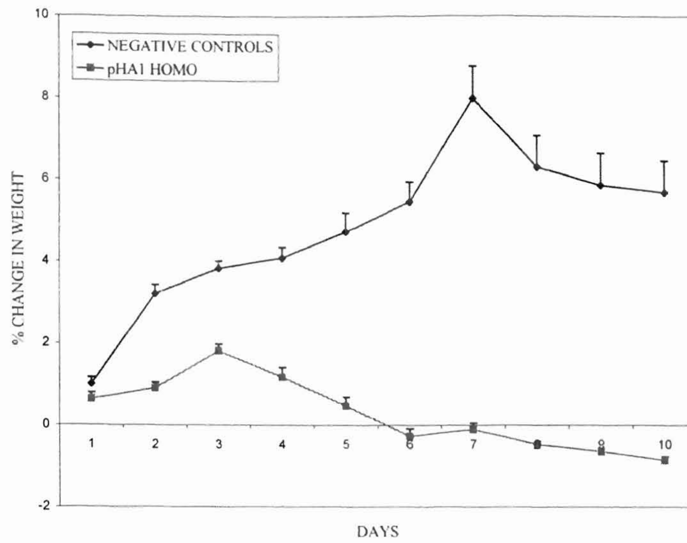

Figure 4 -7C: Post – challenge body weight loss – homologous challenge.

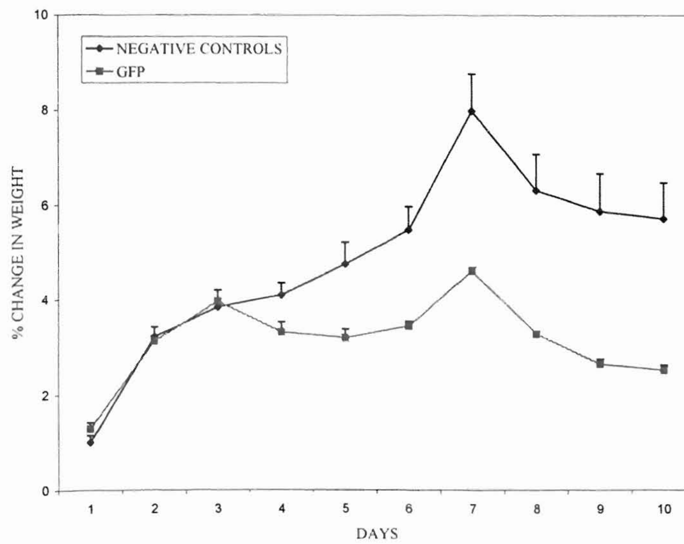

Figure 4 – 7 D: Post – challenge body weight loss – GFP.

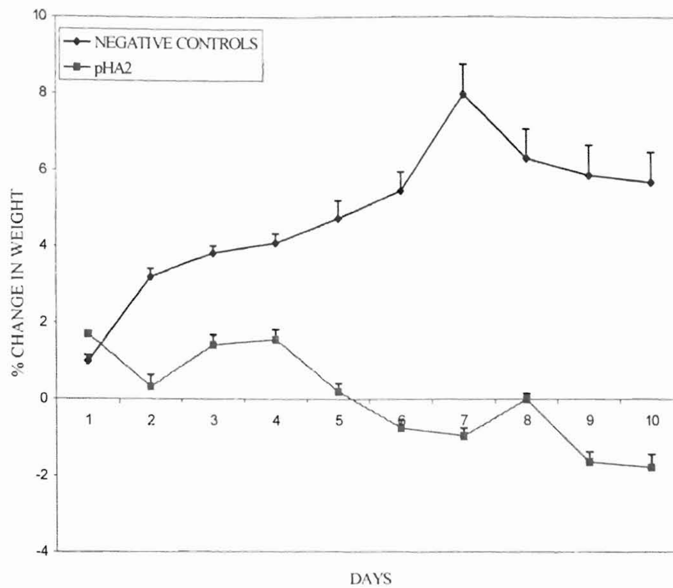

Figure 4 – 7E: Post challenge body weight loss – pHA2.

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From figures 4-7 A-E, it is evident that the vaccinated mice suffered from less severe symptoms and recovered more quickly than the unvaccinated mice. The unvaccinated mice recorded a maximum of 7.9% mean body-weight loss. They did not recover the initial body-weight for more than 10 days. In comparison, the inactivated vaccine group lost a maximum of 1.54%, while the pHA1 and pHA2 groups recorded a maximum loss of 1.8 % and 1.5%, which is comparable to the group immunized with inactivated virus. The pGFP group lost 4.6% that is less than that that of the control group. This may be due to the non-specific protection elicited by the DNA vaccine. The heterologous challenge group recorded a maximum loss of 1.17 % on day 7-post

challenge. The disease was found to persist for a longer period, although the weight loss was not as high as that of the unvaccinated group. This can be attributed to the nonspecific protection elicited by the DNA vaccine (Klinman *et al.*, 1996). Moreover, equine-1 influenza virus, A/Eq/Prague/1/56, may be less virulent to mice than to equine-2 influenza virus A/Eq/Ky/98.

A Student t test was performed on the data. The body weight loss in the vaccinated groups showed a statistically significant difference from that unvaccinated group with p values of P <0.01 while the pGFP group showed a marginal difference with a value of P value of 0.03.

4.4.2 Post - vaccinate and post - challenge sera anti - HA IgG titers:

Post-immunization sera, collected at 21, 35, 50 day, and sera collected 15 days post-infection were assayed for virus-specific antibodies. The isotype of the antibody was determined by ELISA.

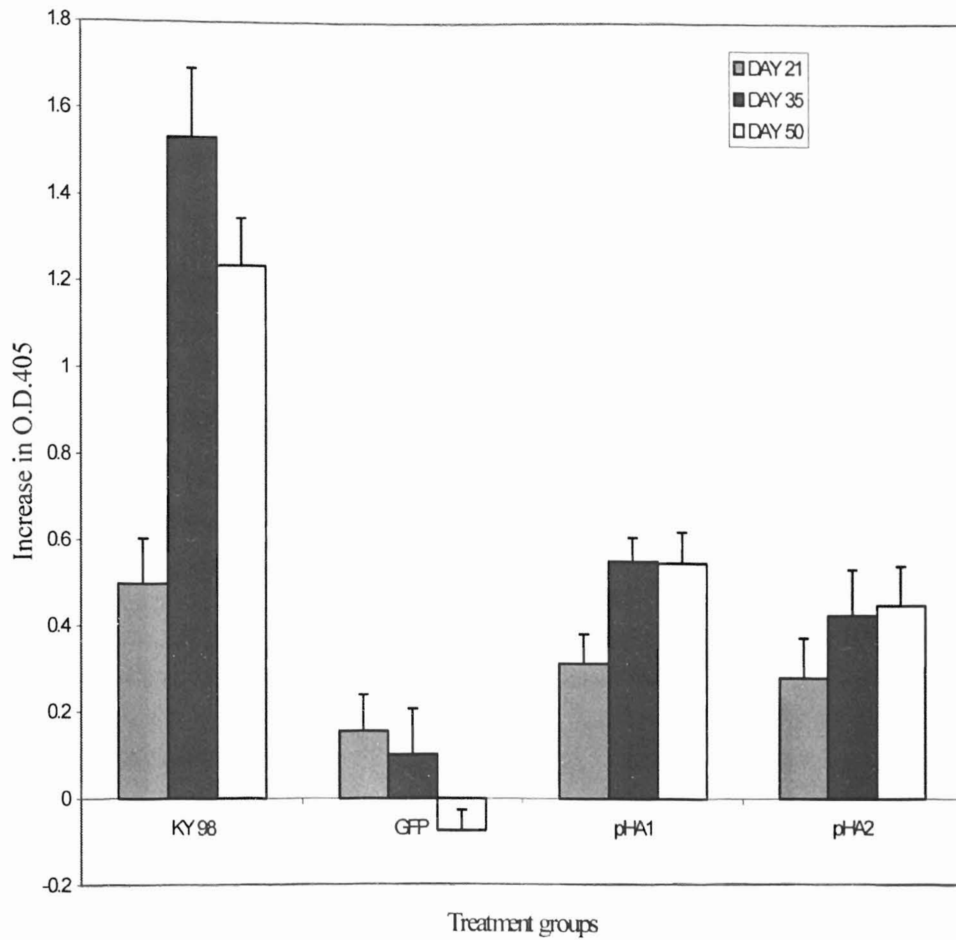

Fig 4-8: Elisa titer of HA specific serum IgG - Post vaccinate serum anti HA IgG titers, showing a titer increase in the vaccinated groups on boosting and maintenance of titers until day 50.

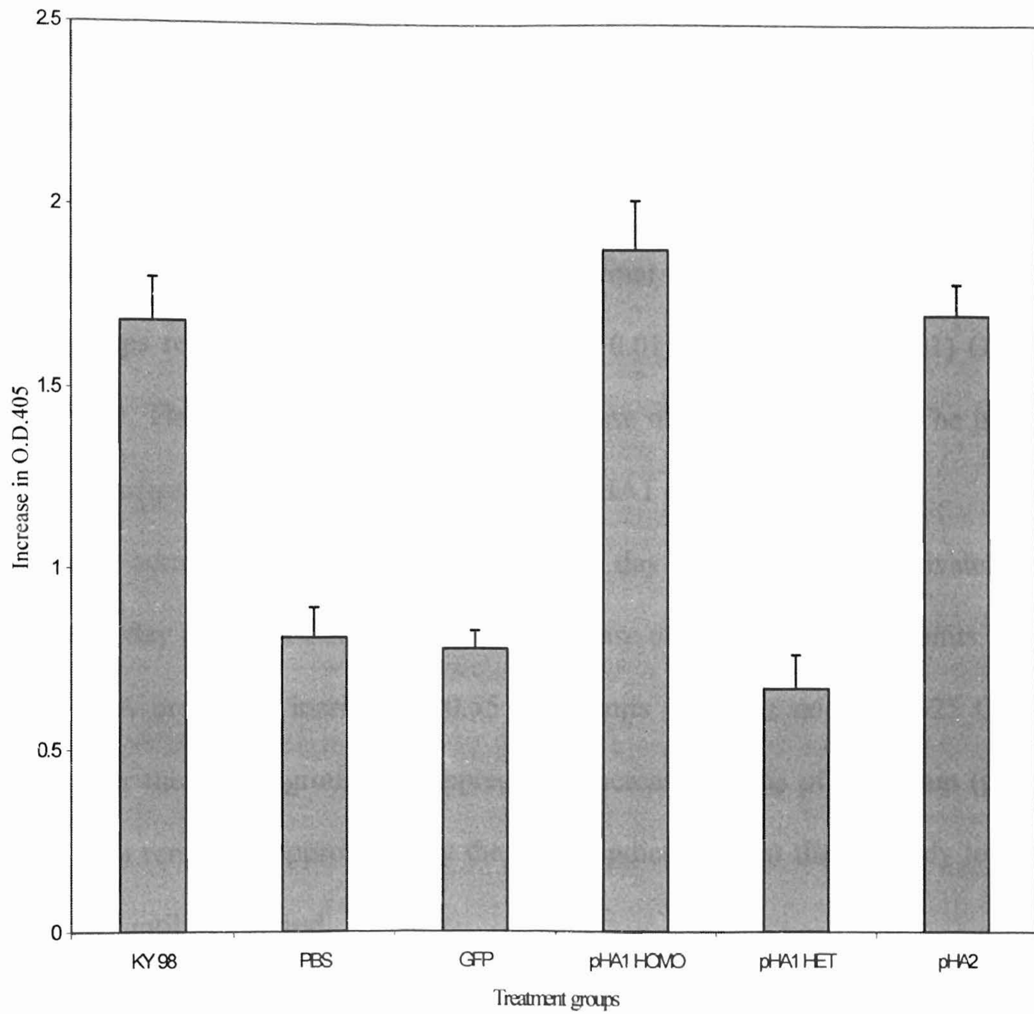

Fig 4-9: Elisa titer of HA specific serum IgG -15th day post challenge – Sera titers show an anamnestic response in vaccinated groups and a primary response in unvaccinated groups.

Post-vaccination and 15 day post virus challenge sera were assayed three times by ELISA to determine the mean increase in serum anti-HA IgG antibodies. Dilutions of 1:10, 1:100, and 1:1000 of the sera were used in the assay. The 1:10 dilution samples yielded an optimal O.D. value. The mean value of three replicates was used in data interpretation and graph construction. The difference in mean values between treatment group sera and the negative control group sera is depicted in the graphs.

On the 21 day post inoculation, the inactivated vaccine group recorded a mean O.D. increase of 0.49 ($p<0.01$) units above normal mouse sera, while the pHA1 and pHA2 groups recorded an increase of 0.31 ($p< 0.01$), and 0.28 ($p = 0.01$) O.D. units, respectively. The pGFP group recorded an increase of 0.156 ($p = 0.35$). The increase in titers is significant for the inactivated virus and pHA1 groups.

After administration of a booster dose on day 21, sera from inactivated vaccine group at 35 day post-inoculation showed an increase of 1.5 ($p<0.01$) O.D. units. Whereas, for the pHA group, an increase to 0.55 O.D. units ($p<0.01$), and to 0.425 O.D. units ($p<0.01$) for the pHA2 group. No appreciable increase for the pGFP group ($p = 0.44$). Day 50 sera remained approximately the same, indicating that the antibody levels were maintained until this period.

On challenge, vaccinated groups demonstrated an anamnestic increase in serum IgG titers while the unvaccinated group mounted a primary response. The O.D. values of the inactivated vaccine group increased to 1.8 O.D. ($p<0.01$) units over normal mouse serum and that of the pHA1 and pHA2 groups increased by 1.8 ($p<0.01$) and 1.7 ($p<0.01$) O.D. units respectively. The heterologous challenge group recorded an increase of 0.672 O.D. units ($p<0.29$), which was not significantly different from the 35th day values. The

tiers of the negative control and pGFP groups increased by 0.81 ($p<0.01$) and 0.78 O.D. units ($p<0.01$) indicating that a primary response was elicited.

4.4.3 Post - vaccinate and post - challenge sera anti - HA IgA titers:

Post-immunization sera, collected at 21, 35, 50 day, and sera collected 15 days post-infection were assayed for virus-specific antibodies. The isotype of the antibody was determined by ELISA.

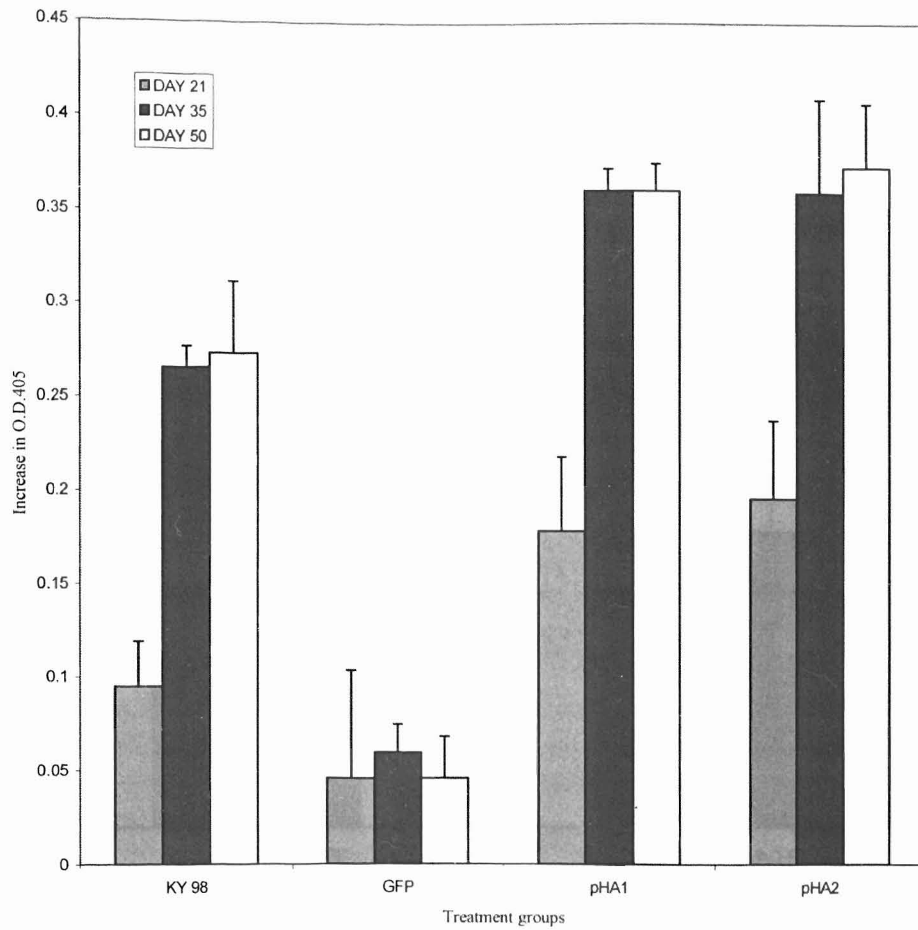

Fig 4-10: Elisa titer of HA specific serum IgA showing an increase in the serum titer of the vaccinated groups on boosting and maintenance of titers until day 50.

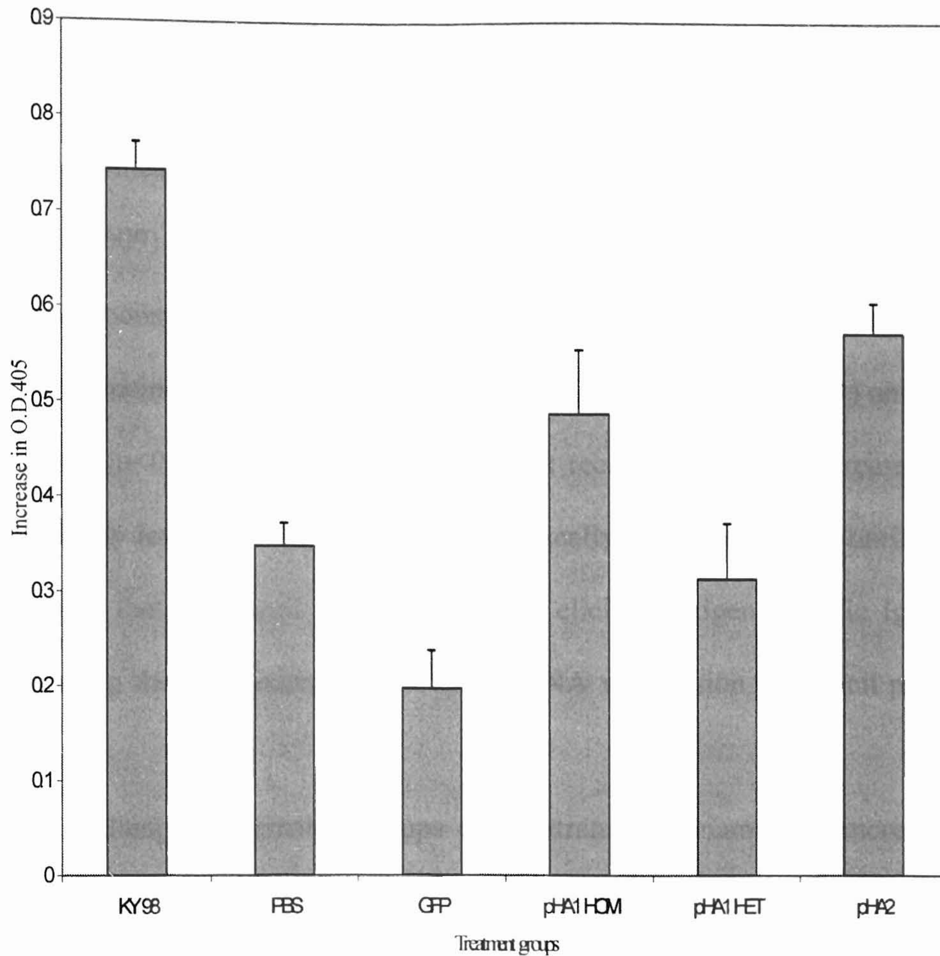

Fig 4-11:15th day post challenge sera anti-HA IgA titers showing an anamnestic response in the vaccinated groups and a primary response in unvaccinated groups.

Post vaccinate and 15th day post-challenge sera samples were assayed three times by ELISA to determine the mean increase in serum anti HA IgA antibodies. Dilutions of 1:10,1:100 and 1:1000 were used in the assay. The 1:10 dilution samples yielded an optimal O.D. value .The mean value of three replicates was used in data interpretation

and graph construction. The difference in mean values between treatment group sera and the negative control group sera is depicted in the graphs.

The 21st day sera samples showed a marginal increase in anti- Ha IgA titers when compared with normal mouse serum. An O.D. increase of 0.09 ($p = 0.01$) was recorded for the inactivated vaccine group, 0.17 for the pHA1 group ($p = 0.01$), and 0.19 for the pHA2 ($p = 0.009$) group and 0.04 for the pGFP group ($p = 0.48$).

Upon booster vaccination on day 21,the values increased to 0.265 for the inactivated vaccine group ($p < 0.01$), 0.36 for the pHA1 group ($p<0.01$) and 0.38 for the pHA2 group ($p<0.01$). The pGFP group did not record significant increase ($p = 0.367$). These antibody levels were maintained at statistically significant levels until day 50. This demonstrated that intranasal DNA vaccination elicited antigen specific IgA responses, thus validating the hypothesis that intranasal DNA vaccination will elicit protective IgA responses.

On challenge, vaccinated groups demonstrated an anamnestic increase in serum IgA titers while the unvaccinated group mounted a primary response. The O.D. values for the inactivated vaccine group increased to 0.74 O.D. ($p < 0.01$) units over that of normal mouse serum, while that of the pHA1 group increased by 0.48 ($p < 0.01$) and the pHA2 group by 0.57 ($p <0.01$). An O.D. increase of 0.34 ($p<0.01$) was recorded for the unvaccinated group and an increase of 0.19 O.D. ($p=0.987$) units was recorded for the pGFP group. An O.D. increase of 0.37($p= 0.749$) was recorded for the heterologous challenge group. This was not a significant difference from the 35th day sera values.

CHAPTER V

CONCLUSIONS

Effective control of influenza is hampered by several factors, like the antigenic shift of the virus and unavailability of effective vaccines. DNA vaccines are a new means of preventing infectious diseases. They have the advantage of being safer than live vaccines and eliciting cell mediated responses like live vaccines. This study is an attempt to evaluate the effectiveness of a DNA vaccine for influenza in eliciting mucosal immunity. The types of immune responses elicited and protection levels afforded by such vaccination were also studied.

5.1 Conclusions:

This study provides evidence that a plasmid encoding the immunogenic influenza HA1 gene, after transfection into mammalian cells, transcribes and translates the recombinant gene, in this case, the HA1 viral protein. Furthermore, intranasal immunization using this DNA vaccine elicits specific IgG and IgA antibodies to the viral protein.

In contrast to other studies--where the entire HA gene sequence, including the HA1 and HA2 segments were incorporated in the DNA vaccine-- this study included only HA1 gene. It is evident that HA1 is sufficient to elicit protective immunity against homologous challenge.

It was found that the intranasal route of delivery of DNA using a liposome vehicle was a very convenient means of administering the vaccine. Dose levels as low as 0.01 ug DNA/gram body-weight is sufficient to elicit protective immunity against sub-lethal challenge in mice when administered by this route. Further, the type of immunity elicited included both IgA and IgG responses. Most other studies involving inoculation by the intramuscular route were has not been very effective in eliciting mucosal immunity (Davis, 2001). Gene-gun vaccination, which is also effective, is impractical under field conditions.

5.2 Significance:

This study provides evidence that DNA vaccination is an ideal means of overcoming the disadvantages associated with currently available inactivated influenza vaccines. It can be expected that this vaccine will prove to be effective in equines. This model can be extended to human influenza vaccines. Difficulties caused by tedious procedures involved in viral adaptation to cell and egg culture, vaccine testing and safety concerns in incorporation of new candidate vaccine strains are eliminated with this type of vaccination. Extraction of the HA gene from the candidate viral strain and incorporation in the DNA vaccine is not a time consuming process.

DNA vaccines can be effective in several respiratory infections. Mucosal immune responses can be elicited by intranasal vaccination. In diseases where a Th1 or Th2 response is preferred, the desired kind of immune response can be targeted by altering the mode of delivery or by incorporation of cytokine encoding genes. This approach is also

useful in the treatment of allergies where a Th2 response should be suppressed to reduce IgE secretion. DNA vaccination will be particularly useful for diseases such as malaria, tuberculosis, and HIV. These diseases are caused by intracellular parasites. Conventional vaccines have not been very effective against these diseases, as they elicit only the humoral immunity. Antibodies do not neutralize intracellular pathogens, as they are inaccessible to the specific antibodies. Another advantage for DNA vaccine is that multi-gene vaccines can be engineered by incorporating immunogenic genes from several pathogens. The number of vaccinations given to an individual or animal will be reduced. This will have a beneficial effect on immunization costs, which is not insignificant for developing countries.

Therefore, DNA vaccines have a vast potential in prevention and treatment of respiratory infections, infections caused by intracellular parasites, allergies, and cancers.

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VITA 2

Sheela Ramamoorthy

Candidate for the Degree of

Master of Science

Thesis: DEVELOPMENT OF A MUCOSAL DNA VACCINE FOR EQUINE INFLUENZA

Major field: Microbiology, Cell and Molecular Biology

Biographical:

Personal Data: Born in Tiruchirapalli, India, October 31, 1967, daughter of J.S.Ramamoorthy and Kamala Ramamoorthy.

Education: Graduated from St.Joseph's Anglo-Indian girl's higher secondary school, Tiruchirapalli, India, in June 1985; received Bachelor of Veterinary and Animal Sciences degree from the Madras Veterinary College, India, in January 1991; completed requirements for the Master of Science Degree At Oklahoma State University in December, 2002.

Professional experience: Teaching Assistant, Department of Microbiology and Molecular Genetics, Oklahoma State University, August 2000 to August 2002.