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DNA Vaccines against Biological Warfare Agents

Final Technology Investment Fund Project Report

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IMPORTANT INFORMATIVE STATEMENTS

This study was conducted in conformity with the Guide to the care and use of experimental animals, published by the Canadian Council on Animal Care.

Abstract

Rapid technological advances in genomics and molecular biology have paved the way for exciting and innovative approaches to produce new generation of DNA-based vaccines. This report summarizes the major findings of the development of DNA vaccines against two potential biological threat agents, western equine encephalitis virus (WEEV) and influenza virus.

DNA vaccination using plasmid DNA encoding the hemagglutinin gene of influenza A virus was found to completely protect mice against a respiratory challenge with a $5 \times LD_{50}$ dose of influenza virus, while all unvaccinated mice succumbed to the infection ($p < 0.001$). When encapsulated in liposomes, the DNA vaccine induced humoral and cell-mediated immune responses, as well as strong mucosal immunity in the respiratory tract, as measured by elevated levels of IgA. When DNA vaccination against WEE virus using a plasmid encoding the structural proteins of the virus was evaluated in mice, the DNA vaccine induced strong protective immunity and protected the animals against an otherwise lethal challenge of a virulent strain of WEE virus.

Together, these results provided a clear proof of concept on the efficacy and safety of DNA vaccines in experimental animals. It is suggested that DNA vaccines may provide a valuable alternatives to live or attenuated vaccines for protection against BW agents.

Résumé

Des progrès technologiques rapides en génomique et en biologie moléculaire ont pavé la voie à des approches novatrices excitantes en vue de la production de vaccins basés sur l'ADN. Ce rapport résume les principales constatations de la mise au point de vaccins à ADN contre deux agents de guerre biologique potentiels, le virus de l'encéphalite équine de l'Ouest (EEO) et le virus de l'influenza.

La vaccination à ADN utilisant de l'ADN plasmidique encodant le gène de l'hémagglutinine du virus de la grippe de type A a protégé complètement des souris de difficultés respiratoires grâce à une dose du virus de l'influenza de $5 \times LD_{50}$ tandis que toutes les souris non vaccinées ont succombé à l'infection ($p < 0{,}001$). Lorsqu'encapsulé dans des liposomes, le vaccin à ADN a induit des réactions immunologiques humorales et à médiation cellulaire ainsi qu'une forte immunité muqueuse de l'appareil respiratoire, telle que mesurée par des niveaux élevés d'IgA. Lorsque la vaccination à ADN contre le virus de l'EEO au moyen d'un plasmide encodant les protéines structurales du virus a été évaluée chez des souris, le vaccin à ADN a induit une forte immunité protectrice et a protégé les animaux contre une dose qui autrement aurait été létale d'une souche virulente du virus de l'EEO.

Ensemble, ces résultats ont fourni une validation de principe claire de l'efficacité et de la sécurité des vaccins à ADN chez des animaux de laboratoire, et donnent à penser que les vaccins à ADN peuvent fournir des solutions de rechange intéressantes aux vaccins vivants ou atténués pour la protection contre des agents de guerre biologique.

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Executive summary

DNA Vaccines against Biological Warfare Agents: Final Technology Investment Fund Project Report

J.P. Wong; L.P. Nagata; M.E. Christophe; F.L. Schmaltz; G. A. Rayner; M.A. Zabielski; DRDC Suffield TR 2003-072; Defence Research and Development Canada – Suffield; August 2003.

Introduction or background: Rapid advances in recombinant DNA technology have resulted in innovative approaches to produce vaccines that are safe, efficacious for use in humans and are cost effective to produce. DNA vaccination (or immunization) refers to an approach whereby a gene from a pathogen is incorporated into a circular piece of DNA (a plasmid) and this is used to induce protective immunity in an animal or human host.

The primary objective of this Technology Investment Fund Project is to evaluate the protection in experimental animals afforded by DNA vaccines for two representative biological threat agent viruses, as a proof of concept experiment demonstration.

Results: The DNA vaccines developed under this project induced protective immune responses in mice and provided excellent protection in the animals against pulmonary challenges with multiple lethal doses of these viruses against influenza A virus and western equine encephalitis virus (WEEV), the representative biological threat agents employed.

In addition, needle-free methods of vaccination using these DNA vaccines were developed and found to afford protection against both influenza virus and WEEV in mice.

Significance: DNA vaccination appears to be a promising and effective means to confer protection against biological threat agents. Unlike some current vaccines in military use which employ live or attenuated pathogens, DNA vaccines do not cause infection; they can also be readily produced in large quantities and do not require refrigeration for storage. Such features make DNA vaccines especially attractive for defence purposes.

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Sommaire

DNA Vaccines against Biological Warfare Agents: Final Technology Investment Fund Project Report

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Introduction ou contexte : : Les progrès rapides de la technologie de l'ADN recombinant ont mené à des approches novatrices en vue de la production de vaccins sûrs, efficaces et économiques. La vaccination (ou l'immunisation) à ADN est une approche dans laquelle un gène d'un agent pathogène est incorporé à une molécule circulaire d'ADN (un plasmide) qui est utilisée pour induire une immunité protectrice chez un hôte animal ou humain.

L'objectif premier de ce projet du Fonds d'investissement technologique est d'évaluer la protection d'animaux de laboratoire fournie par des vaccins à ADN pour deux virus représentatifs des agents de guerre biologique comme expérience de validation de principe.

Résultats : Les vaccins à ADN mis au point dans le cadre de ce projet ont induit des réactions immunologiques de protection chez des souris et ont fourni une excellente protection de ces animaux contre les difficultés pulmonaires avec des doses létales multiples du virus de la grippe de type A et du virus de l'encéphalite équine de l'Ouest (EEO), les agents de guerre biologique utilisés.

En outre, des méthodes de vaccination sans aiguille au moyen de ces vaccins à ADN ont été mises au point et se sont révélées offrir une protection tant contre le virus de la grippe que le virus de l'EEO chez des souris.

Importance : La vaccination à ADN semble être un moyen prometteur et efficace de protection contre les agents de guerre biologique. Contrairement à certains vaccins actuellement en usage par les militaires qui utilisent des agents pathogènes vivants ou atténués, les vaccins à ADN ne causent pas d'infection. Ils peuvent également être produits rapidement en grandes quantités et ne requièrent pas de réfrigération pour leur stockage. Ces caractéristiques rendent les vaccins à ADN particulièrement intéressants à des fins de défense.

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Table of contents

List of figures

Acknowledgements

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1 Introduction

Recent world events indicate that threat from the use of highly pathogenic and deadly microorganisms as biological threat is increasing globally. Vaccination is generally accepted to be the most effective medical countermeasure to protect military and susceptible civilian populations against these agents. However, the deployment of military vaccines has been hindered by concerns about their safety, efficacy, and commercial availability. Most military vaccines against biological warfare agents are either live attenuated (e.g. smallpox virus, *Francisella tularensis* LVS) or inactivated (e.g. western equine encephalitis virus (WEEV), *Yersinia pestis*), and their use has been linked to occurrences of adverse reactions and chronic illnesses [1–5]. These concerns necessitate research and development of novel vaccines that are safe, effective for use in humans, and are cost effective to produce.

The introduction of DNA vaccine technology has resulted in radically new approaches for the production of safer and more efficacious vaccines. This technology takes advantages of rapid advances in recombinant DNA methodologies and increased understanding of microbial genomic sequences. DNA vaccines represent a novel class of vaccines that may offer an exciting new generation of vaccines against potential biological threat agents. DNA vaccination involves the inoculation of a circular DNA containing gene(s) encoding for protective antigens into an animal or human host, with the aim of inducing a long-lasting protective immunity against the microorganism [6–10]. DNA vaccines offer some major advantages over live or inactivated vaccines. DNA vaccines do not cause an infection, carry no risk of reversion to virulence, and are, therefore, superior to live or attenuated vaccines in terms of safety. While most immunizations with conventional vaccines are achieved with needle injections, DNA vaccination can be carried out using particle-mediated or formulated for aerosol or oral delivery, presenting no health risks associated with needle injections. DNA vaccines are particularly suited for military deployment overseas, as they are stable in room temperature and do not require refrigeration. The production of DNA vaccines can be scaled-up using recombinant techniques, and may therefore more readily available than live attenuated vaccines. Due to these desirable attributes, DNA vaccines are rapidly gaining wider acceptance in clinical medicine and a number of the DNA vaccine candidates are in clinical trials [11]. Preliminary results from these trials indicate that these vaccines are effective and can elicit long-lasting protective immunity.

DNA vaccine technology has been largely unexplored for military vaccines. This report explores the development of DNA vaccines for infections by biological threat agent viruses, as well as exploring delivery technologies for needle-free approaches that deliver the vaccines directly to the sites of infection. This report will describe the development of DNA vaccines against 2 viral agents, namely, western equine encephalitis virus (WEEV) and influenza virus.

WEEV is a member of the alphaviruses which include the eastern and Venezuelan equine encephalitis viruses. These viruses are highly infectious and are well suited for aerosol transmission. These viruses are considered to be biological threat agents as they can cause highly incapacitating infections in humans, with case fatality rates ranging from 0.5–40% [12, 13]). There are currently no commercially available vaccines or antiviral drugs against these viruses. An inactivated vaccine to WEEV is under investigational new drug (IND) status. The

vaccine uses formalin-inactivation of cell culture supernatants from WEEV-infected tissue culture. It requires a minimum of 3 doses, yearly monitoring of antibody titer and possible boosters. Its effectiveness in the protection against an aerosol challenge of WEEV has yet to be established. WEEV is endemic in western North America and strains/varieties have been isolated from Argentina (AG80-646), Brazil (BeAr 102091) and the former Soviet Union (Y62-33) [12, 14]. In nature, WEEV is transmitted from its amplifying hosts or reservoir in wild birds, to man and horses, by mosquitoes (*Culex tarsalis* being the principal vector).

While the endemic cycle has resulted in only a limited number of human infections in recent years, in the past, major epidemics of western equine encephalitis (WEE) have been recorded. The most extensive epidemic, including 3336 recognized human cases and 300 000 cases of encephalitis in horses and mules, occurred in the western United States and Canada in 1941 [12, 13].

Influenza viruses are causative agents of influenza pandemics that had killed millions worldwide. The most severe influenza pandemic took place in 1918, when the "Spanish influenza" took more than 20 million lives [15, 16], many of the victims were in their prime of life. In 1957 and 1968, both the Asian and the Hong Kong influenza killed more than one million worldwide. In 1997, a lethal avian influenza virus "cross" species occurred and killed 6 young adults in Hong Kong [17]. These cases were caused by emergence of highly virulent influenza strains. Unless more effective vaccines and new antiviral drugs are available against these influenza variants, the human population is defenseless against similar influenza pandemics in the future. Even in the absence of influenza pandemics, infection and complications from influenza remain a leading cause of human mortality or morbidity globally.

Vaccination using killed whole virus remains the most accepted preventive measure against influenza [18]. However, a major problem with the current influenza vaccines is that they may be ineffective against new variants of the viruses resulting from genetic changes such as antigenic drifts in the hemagglutinin (HA) protein or antigenic shifts to another HA subtypes.

An important component of this project report is the vaccine delivery technologies to increase the efficacy of DNA vaccines. This is important because DNA molecules are particularly susceptible to nuclease degradation in the body, particularly in the serum.

Also, DNA vaccines are large molecules which are charged and, therefore, their ability to cross cell and nuclear membranes is limited. In order to circumvent these problems, liposomes, which are microscopic lipid vesicles used to encapsulate drugs and vaccines, are used as vaccine carriers. When DNA vaccines are encapsulated in liposomes, they are protected from in vivo degradation by nuclease activity [19].

Vaccines encapsulated in liposomes can be taken up efficiently by specific target cells by processes such as phagocytosis and endocytosis, they represent an effective means to enhance transport of DNA vaccines into antigen presenting cells, including dendritic cells. Liposome-encapsulated DNA vaccines can also be delivered using needle-free approaches such as aerosol inhalation. Since the respiratory tract represents the most common route of entry and the primary infection sites for most biological threat agents, aerosol delivery of vaccines will concentrate the protective immunity elicited by the vaccines in the lungs, thereby may significantly enhance the vaccine efficacy.

2 Materials and methods

2.1 Reagents

Lipids used for the preparation of liposomes are 1,2-dioleoyl-3-dimethylammonium chloride (DODAC, Avanti Polar Lipid Inc., Alabaster, AL), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE, Avanti Polar Lipid Inc.), and polyethylene glycol C8 ($PEG_{2000}C_8$CER,Northern Lipid Inc., Vancouver, BC). Plasmid vectors used include pCI (Promega Corporation, Madison, WI), phT3T7BM+ (Boehringer Mannheim, Laval, QC), and pVAX (Invitrogen, La Jolla, CA). Nuclease-free water was from Promega Corporation.

2.2 Animals

Six week old female BALB/c mice were obtained from the mouse breeding colony at DRDC Suffield. The use of these animals was reviewed and approved by DRDC Suffield Animal Care Committee. Care and handling of these animals followed guidelines set out by the Canadian Council on Animal Care.

2.3 DNA vaccine to influenza A virus

Preparation of plasmid DNA (pCI-HA10)

The original HA construct from influenza A/PR/8/34, P8H has been previously described [20]. The HA was excised with HindIII and BamHI followed by subcloning into pT7-6 [21] to give pT76-HA16, which expresses the HA gene from a T7 promoter. The HA gene was re-amplified from the template pT76-HA16 clone using the following primers: HAXba5′, sense (5′ TA*TCTAGA*CAAAAGCAGGGGAAAATAAAACAACCAAAATG 3′); HANot3′, antisense, (5′ AAGTCATA*GCGGCCGC*AAGGGTGTTTTTCCTCAT ATTTCT 3′). The *Xba* I and *Not* I sites in HAXba5′ and HANot′, respectively, are in italics. Amplification of the HA gene was accomplished by PCR (polymerase chain reaction) using the GeneAmp XL PCR kit with rTth DNA polymerase (Perkin Elmer, Foster City, CA), followed by column purification using the QIAquick PCR purification kit (Qiagen, Valencia, CA). The amplified HA gene was further digested with *Xba* I and *Not* I to create the respective sites at the 5′ and 3′ ends. The HA gene was then ligated into the pCI vector (Promega Corporation, Madison, WI) at *Xba* I and *Not* I restriction sites, and transformed into competent *E.coli* DH5a cells (Gibco/BRL, Bethesda, MD). The pCI-HA10 clone was identified as containing the full-length HA gene, by restriction mapping and DNA sequencing (data not shown). *In vitro* transcription and translation of the pCI-HA10 clone was performed using the TNT-coupled system (Promega Corporation) and canine microsomes (Promega), as described by Long *et al*. [22]. Bulk preparations of pCI-HA10 were prepared with the Endofree Plasmid Mega and Giga kits (Qiagen) following manufacturer's directions and analyzed by restriction enzyme digests.

Liposome-encapsulation of pCI-HA10

A number of cationic liposome formulations were evaluated for the entrapment of DNA vaccine to influenza virus. Most of the cationic liposome formulations result in complex formulation with the plasmid DNA, and are therefore not suitable for aerosol delivery. Of the liposome formulations tested, the following formulation was found to result in high entrapment rate:

Liposomes consisting of7% DODAC, 78% 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 15% polyethylene glycol C8 ($PEG_{2000}C_8CER$) were used at 10 mg/mL concentrations. The lipid film was formed at 50 °C using a rotary evaporator (Buchi Rotavapor R110, Brinkman, Rexdale, ON) and then incubated at 50 °C for 2 h under vacuum. The lipid film was reconstituted with distilled water and 1M β-octylglucanopyranoside (OGP, Sigma, Mississauga, ON) detergent at 20% of the total preparation volume. The plasmid DNA was next added to the lipid film at a concentration of 400 μg DNA/mL to 10 mg lipid/mL. The reconstituted preparation was transferred into dialysis tubing (Spectra/Por, MWCO: 12-14,000, Spectrum Laboratories Inc., Rancho Dominguez, CA) and dialyzed in 1X HEPES buffer solution (150 mM NaCl, 20 mM Hepes, pH 7.4) at 23 °C for 15 h. The free, non-encapsulated DNA was removed from encapsulated DNA on a DEAE Sepharose CL-6B (Sigma) anion exchange column. Encapsulation recovery ranged from 38.0% to 57.0% (data not shown). The liposomes preparations were concentrated using Aquacide II (Calbiochem, La Jolla, CA) and polyethylene glycol MW 10,000 (Sigma), and then dialyzed in 1X HEPES for an additional 2 h at 23 °C. Particle size analysis of liposome encapsulated DNA was performed using a Zetasizer 3000 (Malvem Instruments, Point Roberts, WA).

DNA vaccination of mice against influenza A virus

Mice were immunized with naked or liposome-encapsulated plasmid DNA using intramuscular (IM) or intranasal (IN) routes of administration. For intramuscular injection, mice were anesthetized with ketamine:xylazine (50 mg/kg : 50 mg/kg body weight) into the hind leg. A small incision was made exposing the quadricep muscle, and 50 μL of 1 mg/mL DNA preparation was injected slowly. The incision was then sutured. For intranasal administration, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight) by intraperitoneal injection, then 50 μL of 0.4 mg/mL DNA preparation was administered gently into one of the nostrils with a micropipettor. To avoid swallowing of the plasmid into the stomach, the intranasal dosing was given when the animals were completely anesthetized. The applied volume was naturally inhaled into the lungs. Both IM and IN groups received 1–3 additional boosts of DNA, given four weeks apart. One week after each boost, approximately 200 μL of blood was collected via tail bleed and analyzed for anti-HA IgA or IgG by enzyme-linked immunosorbent assay (ELISA).

For animal virus challenge studies, a mouse-adapted strain of influenza A/PR/8/34 (H1N1) was used. The strain was obtained by at least four blind passages in mice using egg-propagated virus (ATCC, Parklawn, MD) as the initial inoculum. The passaging and propagation of this mouse-adapted strain of influenza virus had been previously described in detail [23]. For the vaccine efficacy study, mice immunized with the DNA vaccine were challenged with the virus as described below. A week following the last booster dose, the animals were anaesthetized with sodium pentobarbital (50 mg/kg body weight, IP). When the mice were completely anesthetized, they were inoculated with 50 μL of the egg-propagated

virus by intranasal instillation into the nostrils. The challenge infectious dose was 5 LD_{50} unless otherwise stated. At 14 days post infection, the number of surviving mice in each of the control and test groups was recorded. Ten mice were used in each control and test group.

Characterization of immune responses to DNA vaccine

Mouse-adapted, egg-propagated influenza virus A/PR/8/34 was purified from allantoic fluid by sucrose gradient purification method. Briefly, the influenza virus was precipitated from allantoic fluid with 7% polyethylene glycol and 2.3% sodium chloride with gentle stirring for 15 h at 4 °C. The virus particles were collected by centrifugation at 10,000 g for 30 min at 23 °C. The pellet was re-suspended in phosphate buffered saline (PBS) and layered onto a 20–60% sucrose gradient. After ultracentrifugation at 100,000 g for 4 h at 4 °C, the virus band was isolated and dialyzed in 0.9% saline for at least 3 h at 23 °C. Purified influenza virus was assayed by titration with monoclonal anti-influenza virus type A (HA) antibody (Biodesign International, Saco, ME) to determine the optimum antigen concentration for ELISA. Dilution of 1/20 of the purified influenza virus antigen and coating buffer (15 mM sodium carbonate, 35 mM sodium bicarbonate, 0.02% (w/v) sodium azide, pH 9.6) were used to coat the Nunc Maxisorb flat bottomed 96-well plates (Gibco BRL, Gaithersburg, MD). The plates were sealed and incubated at 4 °C for 15 h. The plates were washed 5 times with 0.1% BSA, 1% Tween 20 in PBS, blocked with of 2% BSA, 1% Tween 20 in PBS for 1 h at 37 °C and incubated with serial dilutions of test mouse sera. After 1 h incubation at 37 °C and washing as described above, the bound antibody was detected by peroxidase-labeled goat anti-mouse IgA or IgG (KPL, Gaithersburg, MD). The peroxidase activity was measured using 2,2′-azino-di[3-ethylbenzthiazoline sulfonate], (KPL) as a substrate and measured at 405 nm after 20 min of incubation at 23°C.

2.4 DNA vaccination to WEEV

Preparation of plasmid DNA against WEEV

The construction of the clones pcDWXH-7 and pCXH-3, encoding the complete 26S region of WEEV strain 71V-1658 has been described [24]. The 26S structural gene insert from pcDWXH-7 was cloned into the mammalian expression vector, pCI (Promega, Madison, WI). Briefly, the pcDWXH-7 plasmid was first linearized using *Hind*III, followed by a Klenow fragment reaction to fill in the 5′ overhang. The insert was then excised using *Xba*I, gel purified, and ligated into the *Xba*/*Sma*I digested pCI vector. The isolated recombinant plasmid, pCXH-3, was characterized as having the correct insert by restriction mapping and DNA sequence analysis. The clone, pcDWXH-7, was digested with *Sac*I and the insert religated in the opposite orientation. The isolate, pcDWHX-45, contained the complete 26S genome of WEEV, with a reversal in the order of cloning sites outside the two *Sac* I sites (*Hind*III on the 5′ end and *Xba*I on the 3′ end). The WEEV 26S gene segment was excised from pcDWHX-45, and cloned into the *Hind*III and *Xba*I sites of the mammalian expression vector, pVAX (Invitrogen, La Jolla, CA). After transformation into *E. coli* DH10α (Invitrogen, Burlington, ON) and screening of inserts by restriction analysis, the resulting isolate, pVHX-6 was identified.

Primer design for DNA sequencing was guided by information from WEEV strain BFS 1703 [24]. Oligonucleotides were synthesized and gel purified either at the Regional DNA

Synthesis Laboratory (Calgary, AB) or on a Beckman Oligo 1000 DNA synthesizer. Automated DNA sequencing was performed using the ABI Prism Dye Terminator Cycle Sequencing or Big-Dye™ Terminator Cycle Sequencing kits of plasmid templates according to the manufacturer's instructions (PE-Applied Biosystems, Foster City, CA). Sequencing reactions were purified on Centri-Sep™ columns (Princeton Separations, Adelphia, NJ), dried, and analyzed on an ABI 310 automated sequencer. Sequence traces generated were edited manually and assembled using the Seqman component of the Lasergene DNA analysis software (DNAStar, Madison, WI). DNA analysis was performed using Lasergene DNA analysis software.

One-step *in vitro* transcription and translation reactions using the TNT™ coupled reticulysate system (Promega Corp.) was used to express the gene products from the insert of both pCHX-3 and pVHX-6 from the upstream T7 promoter. The RNA was translated in the presence of [^{35}S]-methionine to produce radiolabeled WEEV proteins, which could be further processed with the addition to the reaction of canine pancreatic microsomal membranes (Promega Corp.). All components of the in vitro transcription and translation reactions were incubated together for 90 min at 30 °C. Results were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

In a second method, pCXH-3 or pVH-6 plasmid was transfected into Vero or CHO Kl cells using the cationic lipid, Lipofectamine™ (Invitrogen) or Effectene™ (Qiagen, Chatsworth, CA). Briefly, Vero or CHO Kl cells were grown to 50% confluency in Costar Multichamber slides. The monolayers were transfected with pCXH-3 or pVHX-6 in accordance with the manufacturer's directions, followed by a further 24 h incubation after the addition of 5% DMEM. The monolayers were fixed in methanol:acetone (1: 1) for 5 min and washed with PBS containing 0.1 % (v/v) Tween 20 and 3 % BSA (PBS-TB). The cells were incubated 45 min at 37 °C with approximately 10 µg/mL (in PBS-TB) of protein-G purified monoclonal antibodies to the WEEV El (clone 11D2) or E2 (clone 3F3) proteins [28], followed by washing with PBS-TB. Monolayers were incubated with a 1/4000 dilution of goat anti-mouse IgG/lgM (H & L) horseradish peroxidase conjugate (Caltag, San Francisco, CA) for 45 min at 37 °C. After washing with PBS-T, 2 mL of TruBlue™ HRP substrate (Kirkegaard & Perry Laboratories, Gaitherburg, MD) was added, and plates were incubated a further 30 min at RT, followed by microscopic examination.

DNA vaccination of mice against WEEV

Plasmid DNA or an inactivated WEEV virus vaccine in PBS, were administered to the mice by ballistic or intramuscular (IM) routes. For IM administration, a 27 g needle was used to deliver 50 µg of DNA (pCXH-3 or pCI — negative control) or 50 µL of inactivated WEEV vaccine (SALK WEEV inactivated vaccine). The volume of inoculum used was 100 µL, diluted in PBS. 50 µL was administered IM to each of the hind leg muscles of a mouse. When boosters were given, they were administered 14–28 days apart. For ballistic administration, mice were shaved in the abdominal area with electric hair clippers. The mouse was subjected to ballistic delivery of DNA coated onto gold particles following the manufacturer's standard specifications. The Helios Gene Gun (Biorad, Mississauga, ON) was used as directed, at a pressure setting of 400 psi. Mice were given 1.25 µg DNA and 0.5 mg gold, 1 µm diameter, per shot, and up to three shots for one dose time. Boosters were given 14–28 days apart. The mice were challenged 14–28 days after the final booster. Virus challenge was administered to the mice by intranasal (IN) or intraperitoneal (IP) routes. The volumes of inoculum used were

50 μL for IN and 100 μL for IP. For IN administration, mice were anaesthetized with sodium pentobarbital (50 mg/kg body weight intraperitoneal). When the animals were unconscious, they were carefully supported by hands with their nose up, and the virus suspension in PBS was gently applied with a micropipette into the nostrils. The applied volume was naturally inhaled into the lungs. For IP infection, the mouse was manually restrained, and a 1 mL tuberculin syringe fitted with a 27 g needle was used to administer approximately 100 μL of the virus suspension in PBS. Infected animals were observed daily, for up to 14 days post infection

2.5 Statistical analysis

The survival rates in the study between various vaccinated groups and control groups were analyzed by the unpaired t-test. The statistical analysis was done using the GraphPad Prism software program version 2.0 (GraphPad Software, San Diego, CA). Differences were considered statistically significant at $p < 0.05$.

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3 Results

3.1 Construction of plasmid DNA pCI-HA10 against influenza virus

The HA gene used in this study was originally cloned and expressed in the pT76 vector as described previously [20, 21]. The HA was amplified by polymerase chain reaction and the PCR product digested with *Xba*I and *Eco*RI; the fragments were cloned in pCI vector using T_4 DNA ligase. The resultant construct, referred as pCI-HA10, is shown in Figure 1. The pCI-HA10 plasmid was transformed into competent *E. coli* DH5a cells. *In vitro* transcription/translation of the HA product was performed in the presence of canine microsomal membranes and [^{35}S]-methionine, and analyzed by SDS-PAGE and autoradiography (data not shown). The two bands of 82K and 66K may represent the glycosylated and unglycosylated forms of HA protein. The expression of HA by pCI-HA10 was then confirmed by western blot using a specific anti-H1 hemagglutinin monoclonal antibody (results not shown).

3.2 Efficacy of DNA vaccination against influenza A virus infection

The efficacy of naked and liposome-encapsulated pCI-HA10 to protect animals against lethal challenge of influenza virus by intranasal and intramuscular administrations is shown in Figure 2and Figure 3. Non-immunized mice succumbed to the influenza infection as early as 7 days post infection, and all animals were dead by day 9. All mice which received intranasal immunization with naked unencapsulated pCI-HA10 also succumbed to the infection, with no increase in survival rate nor survival time (Figure 2). In contrast, mice immunized intranasally with liposome-encapsulated pCI-HA10 were found to be completely protected, with 100% survival rate ($p < 0.01$ versus control or naked pCI-HA10 group). When the pCI-HA10 DNA was administered by intramuscular injection, both liposome-encapsulated and naked pCI-HA10 plasmid were shown to provide complete protection against the virus challenge (Figure 3). In contrast, liposome-encapsulated pCI without the HA insert provided little or no protection.

3.3 Immunological Responses to DNA vaccination to influenza

Specific IgA titers in serum samples of mice in the various immunized groups were determined by indirect ELISA assay. Mice immunized with liposome-encapsulated pCI-HA10 by intranasal route were found to contain high titers of specific IgA in the sera, while those immunized with naked unencapsulated pCI-HA10 produced only marginally detected levels (Figure 4). Specific IgA antibody was not detected in the serum samples from non-immunized mice or from mice immunized with liposome-encapsulated pCI without the HA insert. Immunization of mice using intramuscular injection of naked or liposorne-encapsulated pCI-

HA10 did not result in any significantly high levels of specific HA IgA. Up to 3 booster injections were intramuscularly given, but no increase in IgA levels was observed (results not shown).

Mice immunized intranasally with liposome-encapsulated pCI-HA10 had a significant increase in serum IgG titers against the HA gene (Figure 5), whereas immunization with naked pCI-HA10 had resulted in no detectable specific IgG.

3.4 Characterization of WEEV DNA vaccine

In the construction of plasmid DNA to WEEV, sequence analysis of the 26S inserts of pCXH-3, and pVHX-6 showed no changes in primary amino acid sequence [24]. *In vitro* transcription and translation of pCXH-3 using TNT T7 rabbit reticulysate system with or without added canine microsomes, demonstrated synthesis of [^{35}S]-methionine-labelled proteins of the correct size, as indicated by western blot and immunoprecipitation with monoclonal antibodies to the NC, El, and E2 proteins [25]. Similarly, the construct pVHX-6 was also demonstrated to produce the proteins of the correct molecular weight, as determined by *in vitro* transcription/translation. Lysates also reacted with monoclonal antibodies to WEEV in western blot and immunoprecipitation analysis (data not shown). Expression of the insert from the CMV promoter was accomplished by transfection of the pCXH-3 or pVHX-6 plasmid into either Vero or CHO Kl cells. Cells expressing the E1 or E2 proteins were detected through the use of specific E1 or E2 monoclonal antibodies to WEEV (Long - mono), followed by histochemical staining with the HRP substrate Tru-Blue™, thus demonstrating the fidelity of the proteins translated and processed from the cloned 26S region by mammalian cells [25].

3.5 Efficacy of DNA vaccination against WEEV infection

Intramuscular administration of pCXH-3 showed partial protection (0–50%), using up to three doses of 50 μg, followed by challenge with WEEV Fleming 3–4 weeks after the final dose (data not shown). The pCXH-3 DNA protected mice when delivered ballistically and using pCI as a control DNA. When two doses of pCXH-3 were given, protection of 50% was demonstrated as compared to no protection for pCI or a single dose of pCXH-3 (Figure 6). Studies examining protection using the pVHX-6 vector, the Gene Gun, and ballistic delivery were promising. Mice were given three doses (2 × 2.5 μg DNA) of control pVAX or vaccine pVHX-6 every two weeks, and then challenged with WEEV Fleming, CBA87, or 71V-1658 two weeks after the final booster.

An inactivated WEEV vaccine control was also included, with mice immunized with 50 μL IM on days 0, 14, and 28, followed by challenge with WEEV Fleming two weeks after the final booster. All the inactivated virus vaccine immunized mice survived, in addition to 100% of the pVHX-6 immunized, 71V-1658 infected group (Figure 7). Although both these groups were completely protected, the pVHX-6 mice showed slight signs of infection (ruffled fur), as compared to no signs of infection in the inactivated virus vaccine immunized mice. The other pVHX-6 immunized mice did not fare as well, with only 60% and 50% mice, respectively, surviving challenge with WEEV Fleming and CBA87. All pVAX control mice succumbed to lethal infection (Figure 7).

4 Discussion

DNA or genetic vaccination using plasmid DNA represents an exciting means of inducing protective immunity against viral infections. This new generation of vaccines provides many advantages over conventional live or killed vaccines. Unlike live or attenuated vaccines, DNA vaccines do not cause infection and, therefore, do not pose inherent safety concerns associated with live or attenuated vaccines. In addition, unlike most subcellular vaccines which induce either humoral or cell-mediated immunity, DNA vaccines can stimulate both humoral and cellular immune responses [6, 7]. Another attractive feature of DNA vaccination is the possibility and flexibility to clone one or more uniquely designed gene sequences of protective antigens into the plasmids. This allows for rational designs of plasmid DNAs to be used in vaccination program against the current strains and subtypes of influenza viruses. Due to the ease of specific gene designs and scale-up procedures afforded by molecular biology, DNA vaccination offers many advantages over conventional live, killed or attenuated vaccines such as stability, safety and design. Due to these promising attributes, research and development of genetic vaccines against biological threat agents is rational, practical and valuable.

Efficient expression of genes in the plasmid DNA encoding the protective antigens requires the physical uptake of the plasmid by the target cells. Although there is ample evidence which suggests that naked plasmid DNA injected directly into the muscles can express the gene of interest and induce protective immunity [6–10], its ability to be taken up by mucosa-associated lymphoid tissues (MALT) and to induce mucosal immunity has not been well documented. Furthermore, needle injection of the DNA vaccines in muscles in humans can be generally painful and may present health-related safety issues such as muscle aches, and inflammation.

Since influenza viruses primarily infect and multiply in the lower respiratory tract, delivery of DNA vaccines to the respiratory tract may result in the induction of a focused long-lasting protective immunity in the lungs. Furthermore, the lymphoid tissues found in the large mucosal surfaces in the respiratory tract may enable mucosal immunity to be induced, thereby may help to prevent the virus attachment of the lung epithelial cells and strengthen the overall immune defence against the infectious virus particles. Results from our present studies suggest the use of vaccine carriers such as liposomes can result in many significant advantages. When intranasal immunization of animals was carried out using pCI-HA10 encapsulated in liposomes, there was a significant enhancement in vaccine efficacy as well as induction of strong mucosal immunity against the expressed gene. Intranasal immunization using naked unencapsulated pCI-HA10 did not provide any significant protection, and did not result in the induction of mucosal immunity. Although naked DNA administered by intramuscular injection can induce strong systemic cellular and humoral immune responses, it is considered to be poor inducer of mucosal immunity [6, 25]. When plasmid DNA is administered into the respiratory tract, liposomes can facilitate the uptake/transport of the plasmid DNA into the induction and effector sites in the bronchus-associated lymphoid tissues (BALT), or the nasal-associated lymphoid tissues (NALT). The delivery of the plasmid DNA by liposomes to these sites can result in the induction of protective mucosal immunity on mucosal surfaces in the respiratory tract. This may account for the observation that liposome-

encapsulated pCI-HA10 in this present study induced strong mucosal IgA response when it is administered into the respiratory tract but did not when injected directly into the muscles. Similarly, intranasal immunization with naked pCI-HA10 did not elicit any detectable mucosal IgA response. The inability of intranasal immunization with naked plasmid DNA to induce mucosal immunity is also reported by others [6]. These findings support the importance of using liposomes as vaccine carriers to the mucosal surfaces. The mucosal surface in respiratory tract is large and represents the primary site of entry and infection for many respiratory pathogens including influenza.

Development of a DNA immunization strategy that could induce protective mucosal immunity would be valuable in reducing morbidity and mortality associated with infections caused by these pathogens [25]. Mucosal vaccination using liposome-encapsulated plasmid DNA could be also very important in eliciting protective immunity at sites distant from site of vaccine administration [25]. Intramuscular immunization with naked and liposome-encapsulated pCI-HA10 provided complete protection in this study, and this may suggest that induction of mucosal immunity is not essential for protective immunity against influenza. However, the dose of pCI-HA10 by intramuscular injection required to achieve complete protection was 2.5-fold higher than intranasal immunization. This suggests mucosal immunity may contribute to the overall protective immunity against respiratory influenza infection.

Alphaviruses, including WEEV, are another class of virus pathogens against which DNA vaccination can afford protection. Whereas influenza viruses infect primarily the lower respiratory tract, alphaviruses cause systemic infections which involve the muscles and central nervous system (CNS), with little pulmonary involvement [27]. In addition, WEEV is transmitted in nature from the amplifying hosts or reservoir in wild birds, to humans or horses via mosquito bites. Since the virus is transmitted from the vector into the host through the skin, the route of DNA vaccine delivery used for WEEV is ballistic (intra-epidermal) delivery of DNA coated onto gold particles, as this route of vaccine delivery mimics the natural route of virus entry.

The use of pCXH-3 in DNA immunization experiments indicated that the construct could partially protect against WEEV intranasal challenge with the Fleming strain, when the vaccine was delivered intramuscularly (Figure 6). The lack of complete protection obtained with these experiments could be due to limited cross-reactivity between the Fleming and 71V-1658 strains. These results are similar to the ballistic delivery ofpVHX-6 or pCHX-3 (Figure 6), where only 50–60% protection was obtained against the Fleming strain. The more rapid rate of killing of the mice may also play a role in the difficulty of providing protection against this virus. Even with the apparent increased level of expression of pVHX -6 in in vitro transcription/translation, the gain is modest when protection against the Fleming strain is undertaken. Nevertheless, the ballistic method of delivery is quick (3 doses over 28 days), and is able to protect completely against the identical strain of WEEV (Figure 7). The cloning and addition of a second strain of WEEV (Fleming) and repeating cross-protection studies, may allow better coverage of a DNA vaccine to WEEV isolates. Of interest, polyclonal mouse ascites to Fleming did not cross neutralize against the 71V-1658 strain (data not shown), adding support to this notion. All mice inoculated with the pVAX control did not survive infection with the three strains of WEEV, indicating the protection obtained was specific for WEEV, and not due to the protective effect of nonspecific adjuvant-like action of CpG motifs [28, 29]. Serum samples taken within 1 week of infection did not show detectable titres (<

1/20) of antibodies to β propriolactone inactivated WEEV B 11 in an ELISA assay (data not shown), indicating a T cell response may be an important component in providing protection.

The plasmids pCXH-3 and pVHX-6 show promise as vaccine candidates for WEEV. This is especially important for protection against an aerosol challenge of WEEV, an event that would be envisioned in a potential biological threat agent attack using WEEV. The reduced protection ofVHX-6 for both the Fleming and CBA 87 strains points to antigenic variation within the WEEV strains. Construction of a second WEEV DNA vector with the Fleming 26S region would likely afford greater cross-protection results for all WEEV strains, as our original assumption that a single strain of WEEV can cross-protect against all WEEV isolates does not appear to hold true. New cross-neutralization and cross-protection studies will be carried out to investigate these possibilities.

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5 Conclusions

Together, the results presented in this study demonstrate the proof of concept that DNA vaccination can elicit protective immunity in the host, and provide effective and complete protection against western equine encephalitis and influenza A viruses. Since safety and efficacy are of paramount importance in vaccines used for the protection of military/civilian populations against biological threat agents, it is suggested that DNA vaccines may represent a newer and safer generation of vaccines which could play an important role in military medicine. Further research into applications of DNA vaccines against bacterial and parasitic threat agents may also be warranted.

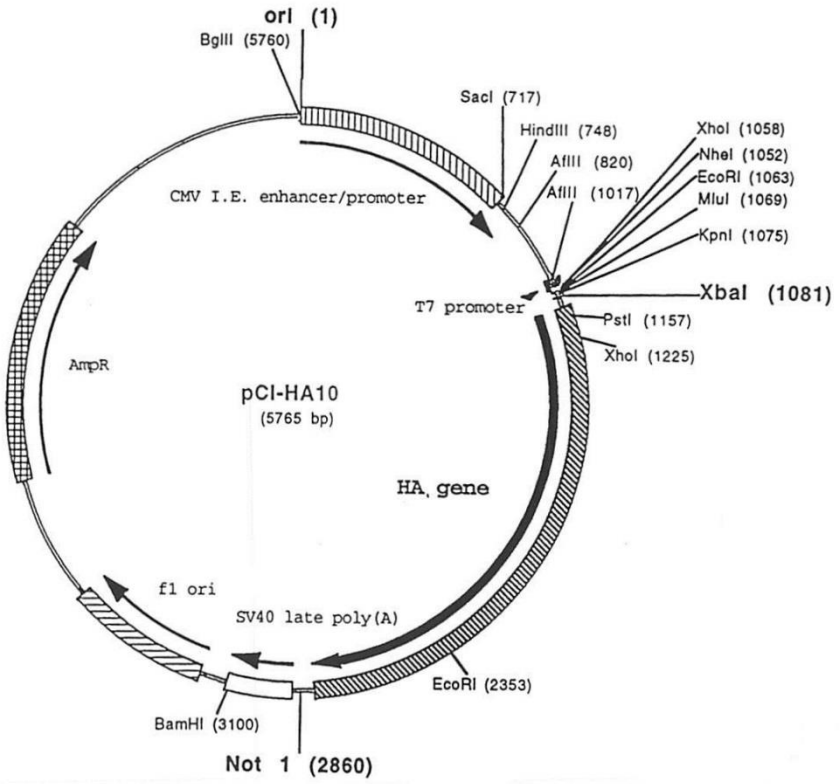

Figure 1.

Figure 1: Schematic representation of pCI-HA10 depicting map of plasmid, cloning and restriction sites and location of HA insert

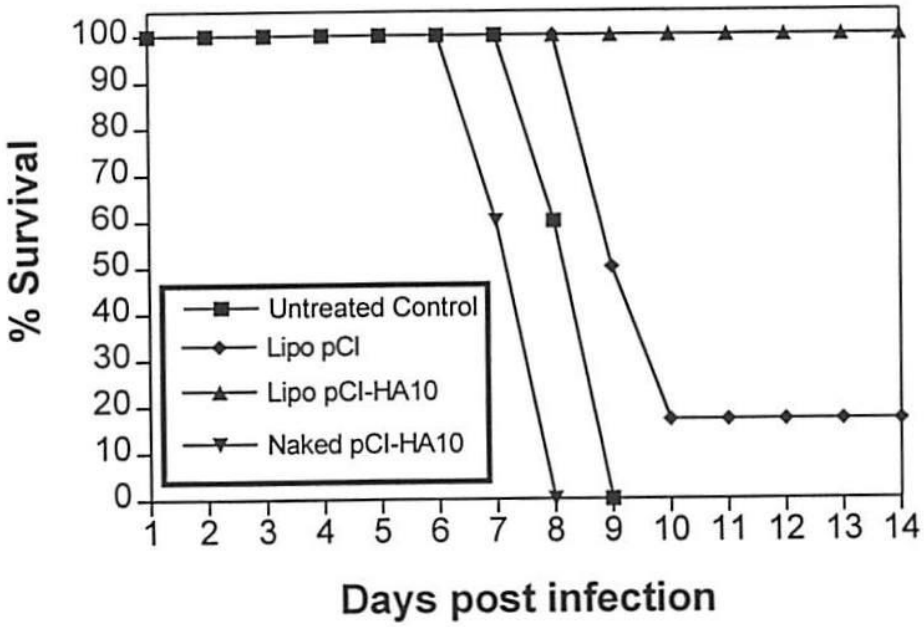

Figure 2 : The efficacy of intranasally administered liposome-encapsulated pCI-HA10 against influenza virus in mice.

Mice intranasally immunized with one primary and 3 booster doses of liposome-encapsulated pCI-HAIO (Lipo pCI-HAIO), naked pCI-HAIO or liposome-encapsulated pCI. At one week post final immunization boost, the mice were intranasally challenged with 5 LDso of virus. The survival rates were monitored daily.

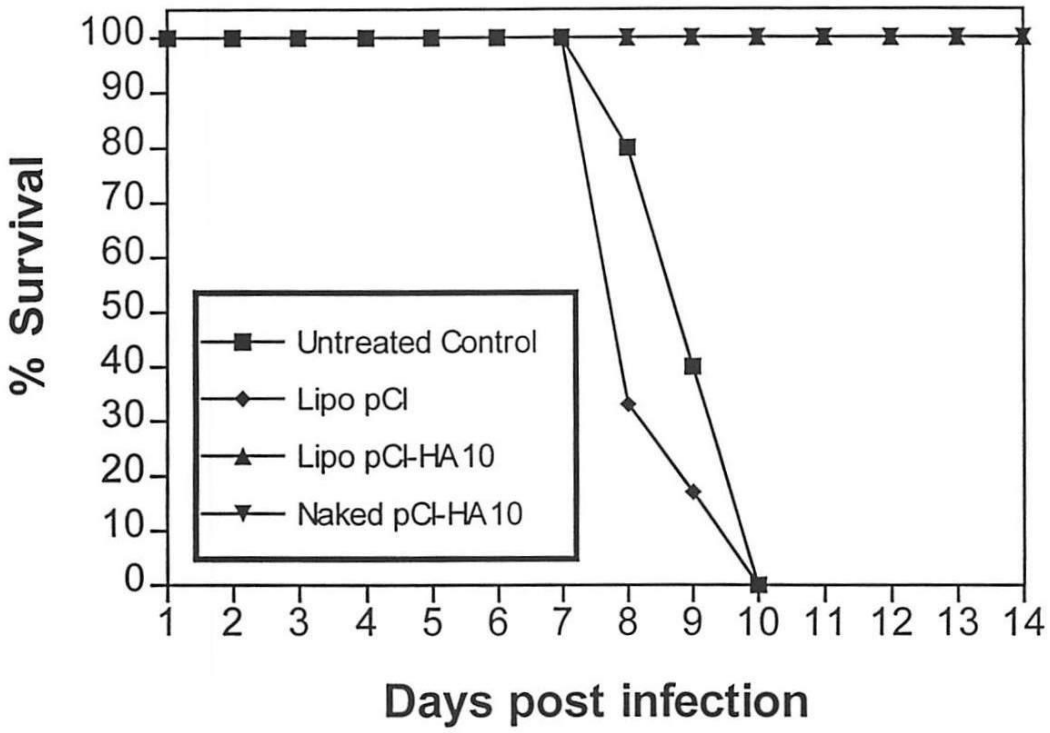

Figure 3 : The efficacy of intramuscularly injected naked and liposome-encapsulated pCI-HA10 to protect mice against respiratory lethal influenza virus challenge

The dosage used was as described for intranasal immunization.

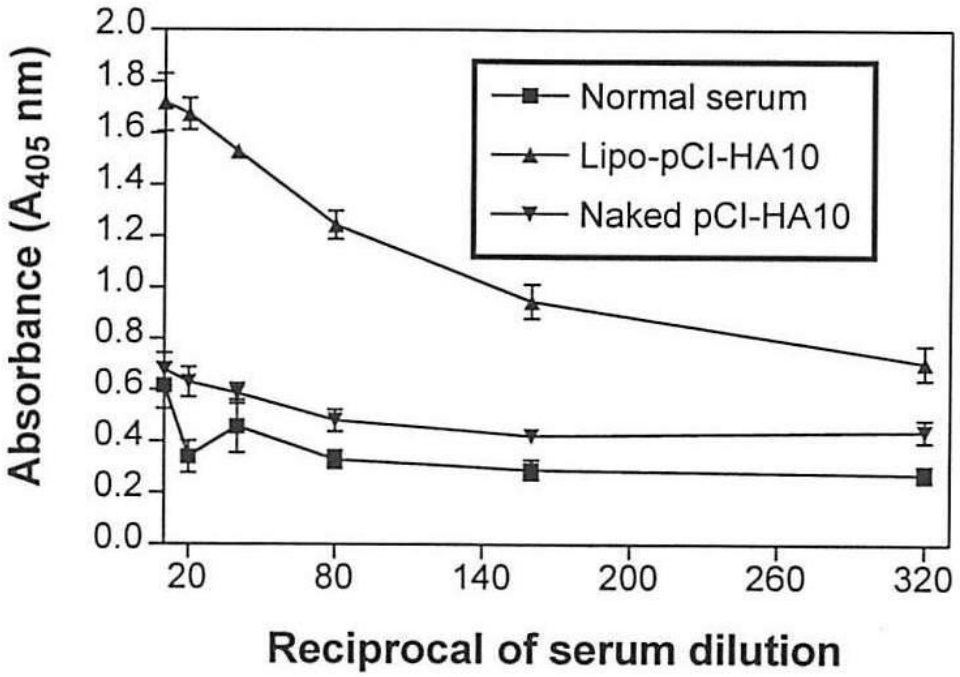

Figure 4 : The IgA levels of mice immunized intranasally with naked and liposomeencapsulated pCI-HA10.

Each mouse received one primary and 3 booster doses of liposome-encapsulated (Lip pCI-HA10) or naked pCI-HA10. At one week post final boost, the animals were tail bled and IgA titers in the serum samples were determined by IgA HA ELISA. Saline controls represent saline used in place of vaccine.

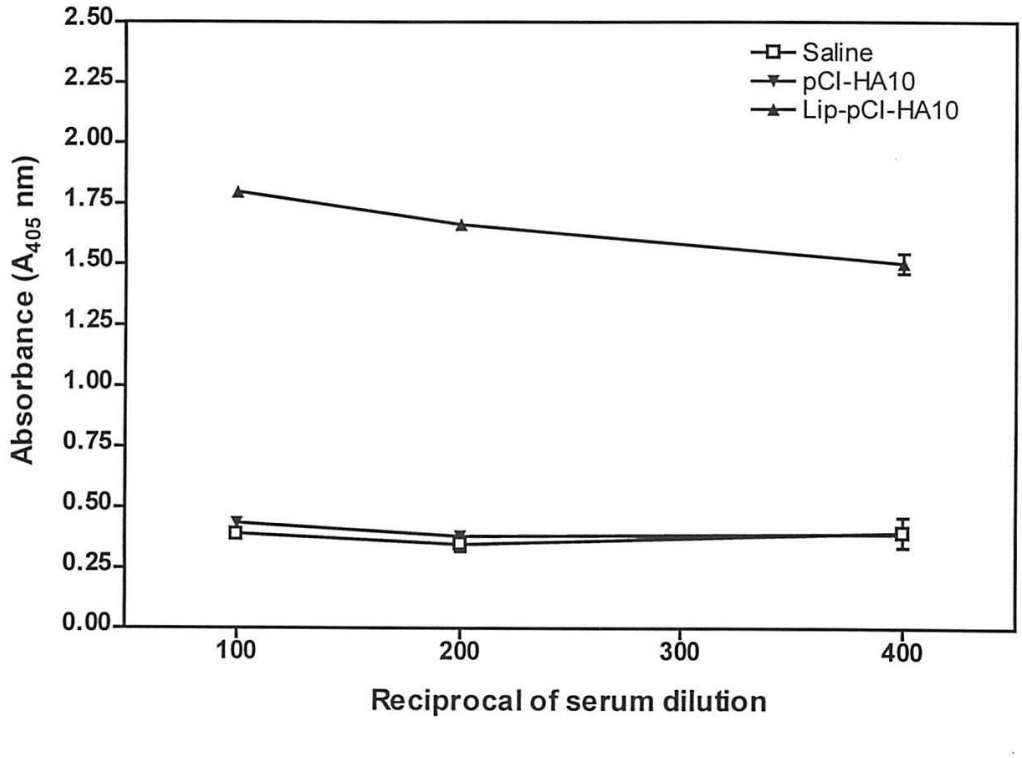

Figure 5 : IgG levels in sera of mice immunized intranasally with naked pCI-HAlO, liposome-encapsulated pCI-HA10, or saline.

Each mouse received one primary and 3 booster doses of liposome-encapsulated (Lip pCI-HA10) or naked pCI-HA10. At one week post final boost, the animals were tail bled and IgG titers in the serum samples were determined by IgG HA ELISA.

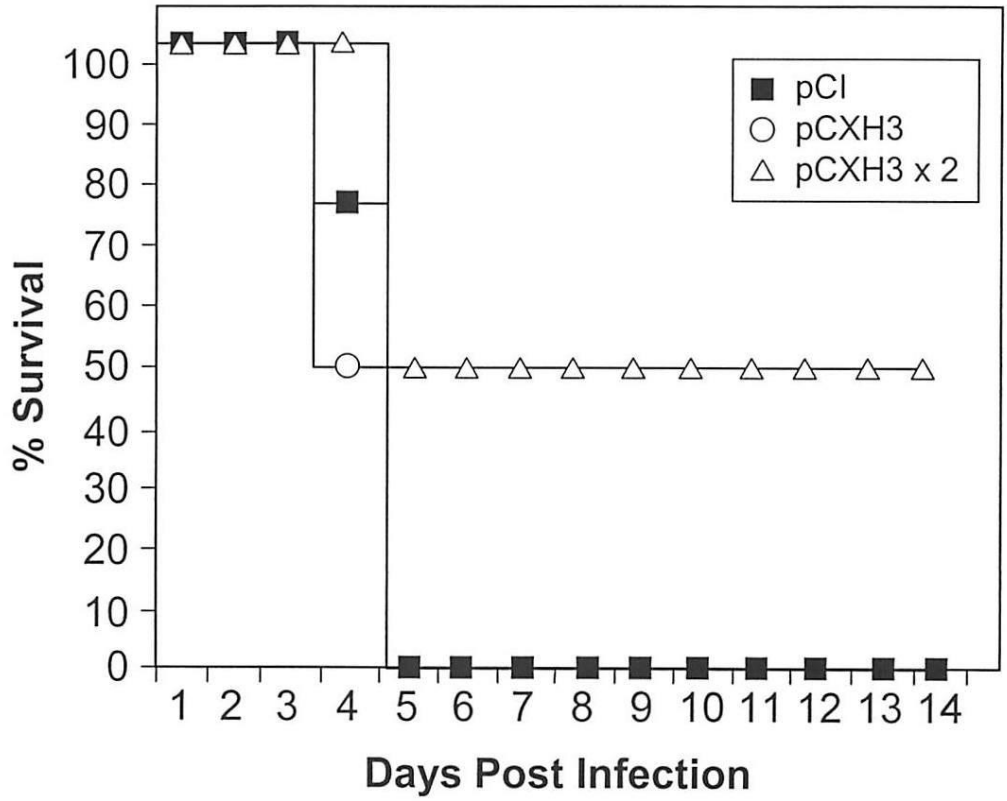

Figure 6 : Efficacy of protection using ballistic delivery of pCXH-3.

Groups of 4 mice were immunized with one or two doses (2 × 1.25 µg) of either pCI control plasmid or pCXH-3 (expressing the WEE structural genes). The interval between boosters (2 doses) or challenge was 3 weeks. The mice were challenged intranasally with 50 µL, of WEE Fleming (1.25 × 104 PFU). The mice were monitored for 12 days and the percent survival graphed.

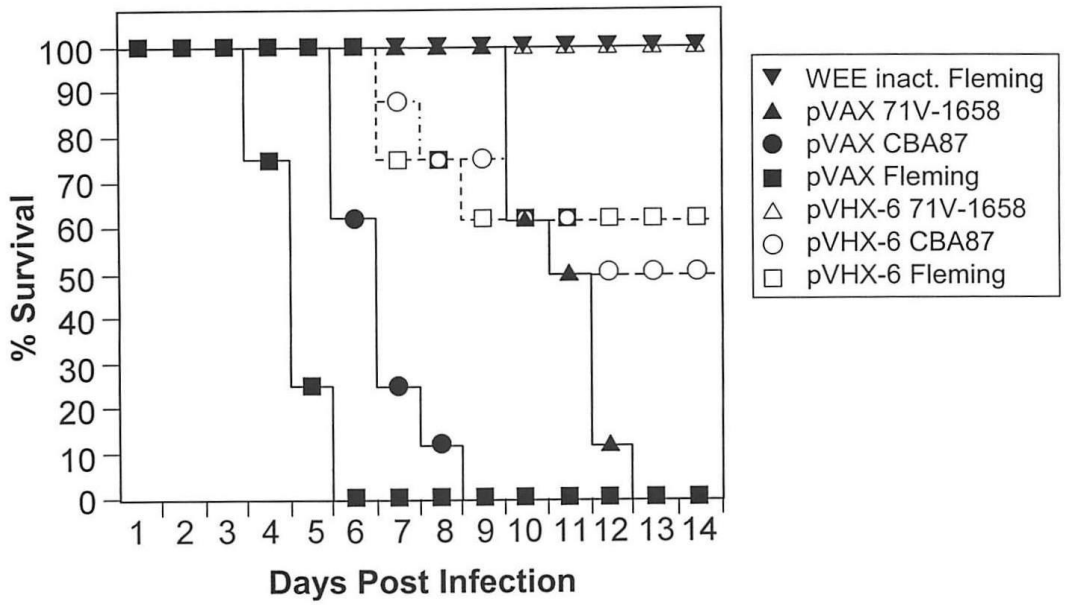

Figure 7 : Efficacy of protection using ballistic delivery of pVHX-6 against WEE.

Groups of 8 mice were immunized with four doses (2 × 2.5 µg) of pVAX control plasmid or pVXH-6. The interval between boosters or challenge was 2 weeks. The mice were challenged intranasally with 50 µL of WEEV Fleming, CBA 87 or 71V-1658 (1.5 × 103 PFU). The mice were monitored for 14 days and the percent survival graphed.

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List of symbols/abbreviations/acronyms/initialisms

DNA	deoxyribonucleic acid
DND	Department of National Defence
IM	intramuscular
IN	intranasal
IP	intraperitoneal
LD_{50}	lethal dose for 50% of a population
PBS	phosphate-buffered saline
WEEV	western equine encephalitis virus

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Rapid technological advances in genomics and molecular biology have paved the way for exciting and innovative approaches to produce new generation of DNA-based vaccines. This report summarizes the major findings of the development of DNA vaccines against two potential biological threat agents, western equine encephalitis virus (WEEV) and influenza virus.

DNA vaccination using plasmid DNA encoding the hemagglutinin gene of influenza A virus was found to completely protect mice against a respiratory challenge with a $5 \times LD_{50}$ dose of influenza virus, while all unvaccinated mice succumbed to the infection ($p < 0.001$). When encapsulated in liposomes, the DNA vaccine induced humoral and cell-mediated immune responses, as well as strong mucosal immunity in the respiratory tract, as measured by elevated levels of IgA. When DNA vaccination against WEE virus using a plasmid encoding the structural proteins of the virus was evaluated in mice, the DNA vaccine induced strong protective immunity and protected the animals against an otherwise lethal challenge of a virulent strain of WEE virus.

Together, these results provided a clear proof of concept on the efficacy and safety of DNA vaccines in experimental animals. It is suggested that DNA vaccines may provide a valuable alternatives to live or attenuated vaccines for protection against BW agents.

Des progrès technologiques rapides en génomique et en biologie moléculaire ont pavé la voie à des approches novatrices excitantes en vue de la production de vaccins basés sur l'ADN. Ce rapport résume les principales constatations de la mise au point de vaccins à ADN contre deux agents de guerre biologique potentiels, le virus de l'encéphalite équine de l'Ouest (EEO) et le virus de l'influenza.

La vaccination à ADN utilisant de l'ADN plasmidique encodant le gène de l'hémagglutinine du virus de la grippe de type A a protégé complètement des souris de difficultés respiratoires grâce à une dose du virus de l'influenza de $5 \times LD_{50}$ tandis que toutes les souris non vaccinées ont succombé à l'infection ($p < 0{,}001$). Lorsqu'encapsulé dans des liposomes, le vaccin à ADN a induit des réactions immunologiques humorales et à médiation cellulaire ainsi qu'une forte immunité muqueuse de l'appareil respiratoire, telle que mesurée par des niveaux élevés d'IgA. Lorsque la vaccination à ADN contre le virus de l'EEO au moyen d'un plasmide encodant les protéines structurales du virus a été évaluée chez des souris, le vaccin à ADN a induit une forte immunité protectrice et a protégé les animaux contre une dose qui autrement aurait été létale d'une souche virulente du virus de l'EEO.

Ensemble, ces résultats ont fourni une validation de principe claire de l'efficacité et de la sécurité des vaccins à ADN chez des animaux de laboratoire, et donnent à penser que les vaccins à ADN peuvent fournir des solutions de rechange intéressantes aux vaccins vivants ou atténués pour la protection contre des agents de guerre biologique.

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DNA vaccines; western equine encephalitis virus; influenza virus; biological warfare agents