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TOWARDS THE DEVELOPMENT OF DNA VACCINE AGAINST NEWCASTLE DISEASE VIRUS

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TOWARDS THE DEVELOPMENT OF DNA VACCINE AGAINST NEWCASTLE DISEASE VIRUS

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The etiological agent of Newcastle disease, Newcastle disease virus (NDV), a member of the family *Paramyxoviridae* and the genus of *Rubulavirus*, can cause up to 100% morbidity and mortality. Immune responses to both the fusion (F) and haemagglutinin-neuraminidase (HN) protein antigens of NDV were demonstrated to play an important role in the prevention of infection. Towards development of DNA vaccine, both the F and HN genes of a Malaysian heat resistant viscerotropic-velogenic NDV strain AF2240 were amplified and cloned into a mammalian expression vector, pEGFP-Ns, and expressed in a mammalian cell line under the control of the immediate early promoter of human cytomegalovirus.

Six recombinant plasmids were constructed, namely pEGFP-N3/F, -N1/HN, -N3/HN-GFP, -N2/Fkoz, -N2/HNkoz and –N1/Fkoz-GFP with the later three constructs introduced with the *kozak* translation initiation sequences. Transient expression of F and HN proteins was assayed in vitro in Vero cell at 48 h post-transfection by indirect immunofluorescence using NDV polyclonal antibody and

fluorescein isothiocyanate (FITC)-labelled anti-chicken IgG. The results showed that all the DNA-transfected cells exhibited bright cytoplasmic fluorescene, indicating both the F and HN proteins were successfully expressed in the mammalian cell line. Immunoblot analysis of the transfected cell lysates further verified the presence of the recombinant proteins with a distinct band of 64 kDa which corresponds to the uncleaved precusor F_o glycoprotein of NDV and two bands of ~62 and 72 kDa as unglycosylated and glycosylated HN glycoproteins, respectively. [^{35}S]-methionine pulsed labelling of transfected cells confirmed the expression of green fluorescent protein (GFP)-fusion protein of F, but not HN-GFP.

DNA inoculation in Balb/c mice and specific pathogen free (SPF) chicken revealed that the efficacy of DNA vaccines could be boosted by co-administration of Freund's adjuvant and repeating DNA immunization. The vaccine trial in SPF chickens showed that both the circular and linearized plasmid DNA of pEGFP-N3/F produced significant levels of antibody against NDV after the second booster and conferred 40-47% protection upon lethal NDV challenge. Co-administration of the circular plasmids of pEGFP-N3/F and –N1/HN, produced antibodies efficiently and conferred more than 50% protection upon NDV challenge. The low and undetectable antibody level in some of the survivors suggests that DNA vaccine elicits cellular immune response in chicken. The overall results also suggest that both the F and HN-DNA can be used as a vaccine component to provide effective protection against NDV and DNA immunization opens a new approach to the development of gene vaccine for chicken against infectious disease.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah untuk Doktor Falsafah

MENUJU KE ARAH PERKEMBANGAN VAKSIN DNA UNTUK VIRUS PENYAKIT NEWCASTLE

Oleh

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Agen etiologi penyakit Newcastle, iaitu virus penyakit Newcastle (NDV), anggota famili *Paramyxoviridae* dan genus *Rubulavirus*, boleh menyebabkan 100% morbiditi dan mortaliti. Reaksi keimunan terhadap antigen-antigen protein pertaupan (F) dan hemaglutinin-neuraminidase (HN) NDV memainkan peranan yang penting dalam pencegahan jankitan. Untuk menghasilkan vaksin DNA, kedua-dua gen F dan HN strain AF2240 NDV Malaysia yang velogenik viserotropik telah diamplifikasikan and diklonkan ke dalam vector pengekspresan mamalia, pEGFP-Ns, dan diekspreskan dalam sel mamalia di bawah pengaruh promoter sitomegalovirus manusia.

Enam plasmid rekombinan telah dibina, iaitu pEGFP-N3/F, -N1/HN, -N3/HN-GFP, -N2/Fkoz, -N2/HNkoz dan –N1/Fkoz-GFP dengan ketiga-tiga plasmid terakhir disertakan dengan jujukan permulan penterjemahan *kozak*. Pengekspresan sementara bagi protein-protein F and HN telah dieseì secara in vitro dalam sel Vero 48 jam selepas proses transfeksi oleh imunopendarfluoran dengan menggunakan antibodi poliklonal NDV dan IgG anti-ayam berlabel pendarfluoran isothiosinat (FITC).

Keputusan menunjukkan kesemua sel yang ditranfeksikan DNA memberi pendarfluor sitoplasmik, menandakan kedua-dua protein F dan HN telah diekspreskan dalam sel mamalia. Analisis imunoblot bagi lisat sel transfeksi menunjukkan kahadiran jalur protein rekombinan pada 64 kDa yang berpadan dengan protein prekursor F_o NDV tidak terpotong dan dua jalur protein pada ~62 dan 72 kDa sebagai HN glikoprotein yang tidak berglikosilasi dan berglikosilasi masing-masing. Sel-sel transfeksi yang berlabel dengan $[^{35}S]$-methionin menunjukkan pengekspresan protein pendarfluoran hijau (GFP) bertaupan dengan F, tetapi bukan GFP-HN.

Inokulasi DNA dalam tikus Balb/c dan ayam pathogen bebas spesifik (SPF) menunjukkan keefikasian vaksin DNA boleh ditingkatkan dengan penyuntikan bersama adjuvan Freund dan imunisasi DNA berulang. Percubaan vaksin dalam ayam SPF menunjukkan bahawa kedua-dua plasmid DNA bulatan dan terpotong bagi pEGFP-N3/F menghasilkan antibodi setelah suntikan booster kedua and memberi perlindungan 40-47% terhadap saingan NDV. Penyuntikan bersama plasmid-plasmid pEGFP-N3/F dan –N1/HN menghasilkan antibodi yang berkesan dan memberi lebih daripada 50% perlindungan terhadap jangkitan NDV. Tahap antibodi yang terlalu sedikit dalam ayam-ayam yang masih hidup mencadangkan bahawa vaksin DNA dapat menghasilkan tindakbalas keimunan sel dalam ayam. Keputusan keseluruhan juga mencadangkan bahawa kedua-dua DNA F and HN boleh digunakan sebagai komponen vaksin untuk memberi perlindungan efektif terhadap jangkitan NDV dan imunisasi DNA membuka satu pendekatan baru dalam perkembangan vaksin gen untuk ayam daripada dijangkiti penyakit.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

A_{260}	-	absorbance at 260 nm
Ag	-	antigen
APCs	-	antigen presenting cells
APS	-	ammonium persulfate
bp	-	base pair
BCP	-	1-bromo-3-chloro-propane
BSA	-	bovine serum albumin
cDNA	-	complementary deoxyribonucleic acid
Ci	-	curie
CMI	-	cell-mediated immune
CTL	-	cytotoxic T lymphocyte
dATP	-	deoxyadenosine triphosphate
dCTP	-	deoxycytidine triphosphate
ddATP	-	dideoxyadenosine triphosphate
ddCTP	-	dideoxycytidine triphosphate
ddGTP	-	dideoxyguanosine triphosphate
ddNTP	-	dideoxynucleotide triphosphate
ddTTP	-	dideoxythymidine triphosphate
DEPC	-	diethylpyrocarbonate
dGTP	-	deoxyguanosine triphosphate
DMSO	-	dimethy sulfoxide
DNA	-	deoxyribonucleic acid
DNase	-	deoxyribonuclease

dNTP	-	deoxynucleotide triphosphate
DTT	-	dithiothreitol
dTTP	-	deoxythymidine triphosphate
EDTA	-	ethylenediaminetetraacetic acid disodium salt
EGFP	-	enhanced green fluorescent protein
ELISA	-	enzyme-linked immunosorbent assay
F	-	fusion gene/protein
FITC	-	fluorescein isothiocynate
g	-	gravity
h	-	hour
HI	-	haemagglutination inhibition
HN	-	haemagglutinin-neuraminidase
HRP	-	horseradish peroxidase
IE	-	immediate early
IFN	-	interferon
Ig	-	immunoglobulin
IL	-	interleukin
kb	-	kilobase
kDa	-	kilodalton
Mab	-	monoclonal antibody
MCS	-	multiple cloning site
MHC	-	major histocompatibility complex
M*r*	-	relative molecular weight
mRNA	-	messenger ribonucleic acid
NDV	-	Newcastle disease virus

OD	-	optical density
ORF	-	open reading frame
PBS	-	phosphate-buffered saline
P_{CMV}	-	cytomegalovirus promoter
PCR	-	polymerase chair reaction
PVDF	-	polyvinylidene difluoride
RBC	-	red blood cell
RNA	-	ribonucleic acid
RNase	-	ribonuclease
RT-PCR	-	revese transcriptase-polymerase chain reaction
s	-	second
SDS-PAGE	-	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIg A	-	secretory immunoglobulin A
SPF	-	specific pathogen free
SV40	-	simian virus 40
Taq	-	*Thermus aquaticus*
TBE	-	Tris-boric-EDTA buffer
TCR	-	T cell receptor
TEMED	-	*N,N,N',N'*,-tetramethylethylenediamine
Th	-	T helper
Tris-HCl	-	Tris hydrochloride
UV	-	ultraviolet
X-gal	-	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

CHAPTER I

INTRODUCTION

Background

Newcastle disease (ND) continues to be one of the most important constraints to the development of improved livestock production in both developing and developed countries (Awan *et al*., 1994; Maldonado *et al*., 1994; Alexander, 1995). At present, animal husbandry and production industries, particularly in the developing countries, are attempting to meet the demands for increased food production from expanding human populations. In Peninsular Malaysia, the poultry industry has developed to a most scientifically advanced industry and contributes more than 60% of the total value of livestock. In order to maintain and increase the productivity of poultry products, both the vaccination and therapeutic or prophylactic use of drugs play an important role in animal disease control. Now, vaccination is increasingly being viewed as the most sustainable option, as it has a major impact on the control of epidemic viral disease of livestock such as foot-and-mouth disease (Bachrach *et al*., 1975; Di Marchi *et al*., 1986) and rinderpest (Romero *et al*., 1993). Moreover, it has the potential to offer for greater economic efficiency in every aspect.

Vaccination against ND virus (NDV) is a common practice worldwide in the poultry industry (Beard and Hanson, 1984; Glisson and Kleven, 1993). In the past decades, protection from ND has traditionally relied on the use of either attenuated or killed vaccines. The most widely used vaccines are live viruses consisting of either lentogenic or selected mesogenic strains propagated in embryonated chicken eggs. The most common NDV vaccine strains are V4, Ulster 2C, Hitchner B1, Asplin F and La Sota (all lentogenic) and Roakin, Mukteswar and Komarov (all mesogenic) (reviewed in Alexander, 1988). Both the attenuated and killed vaccines have been used successfully to induce protective level of immunity among the poultry not only in Malaysia, but also in Australia and many other parts of the world where endemic NDV is prevalent (Alexander, 1995). Nonetheless, problems may arise in field when insufficient attention is paid to factors such as age of the birds, route of inoculation, the strains of virus and follow-up serology (Taylor *et al*., 1990; Glisson and Kleven, 1993). For live attenuated vaccine, there is always a degree of unpredictability as it may cause clinical disease if not attenuated sufficiently (Mckee *et al*., 1987) and most importantly, the possibility of reversion to a more virulent potentially disease-causing phenotype which can establish persistent or latent infection to the host (Weeks-Levy *et al*., 1991; Ogra *et al*., 1991). In addition, live recombinant vaccine, which is engineered to express recombinant genes of which its products are immunogenic, is able to induce both humoral and cell-mediated responses, however, it may not be safe for immunocompromised individuals such as AIDS patients. On the other hand, killed vaccines are generally safe, but unable to generate protective levels of immunity for reasons of losing important epitopes of antigen during inactivation (reviewed in Cox *et al*., 1993). Moreover, multiple boosters are usually necessary in order to generate continual antigen exposure. Furthermore, killed vaccines do not sustain in the host or enter into the host cell, therefore, do not synthesize endogenous protein and unable to induce cytotoxic T cells (Monaco, 1992), possibly a desirable property of an effective vaccine (Leung and Ada, 1982; Taylor and Askonas, 1986). In addition, killed vaccines also have potential risk in which incomplete or improper killing of virus could result in the contamination of vaccines with active wild type virus, especially during large-scale production of the virus (reviewed in Kang, 1989).

In the early 1980s, the advent of recombinant DNA technology created excitement and provided new opportunities to produce vaccines based on the use of expressed products of cloned genes. The approach of using purified recombinant proteins or subunit protein vaccines, consisting of non-replicating and non-infectious portions of the pathogenic agent, has provided some solutions to problems such as incomplete inactivation, unsatisfactory attenuation of the virus and the possible biological contamination of the vaccines that may occur during large-scale production of virus. The use of purified recombinant proteins for vaccination has shown success where the immunity has been achieved with antigen produced in *Escherichia coli*, yeast or other eukaryotic systems (McAleer *et al.*, 1984; Willadsen *et al.*, 1989; Musoke *et al.*, 1992). Recently, the advent of expression vector such as baculoviruses, which can produce large quantities of desired proteins in cell culture, has greatly enhanced the possibilities for other subunit vaccine development (Miller, 1988; Marshall and Roy, 1990; Pearson and Roy, 1993). Undoubtedly, subunit protein vaccines are safe to use. However, they are easily denatured during purification and are not always in their native form when introduced into the host (Finn, 1998), hence, making them poorly immunogenic.

Despite the effectiveness of the current vaccines, many such vaccines are still inadequate for the reasons of safety, cost effectiveness and efficacy related to the risk of infection from the emergence of vaccine-escape mutant (Carman *et al.*, 1990). Therefore, further development of new vaccines to improve the efficacy of vaccination is desirable. At present, direct gene transfer into mammalian somatic tissue *in vivo* is a powerful approach for gene therapy with potential application. Within a decade, the field of genetic vaccination has been studied and developed rapidly and has taken on a new urgency as the vulnerability of related populations to infectious disease increases. The development of DNA vaccine against viral infection has become a great surprise and challenge in the molecular medicine after its first introduction by Wolff *et al.* in 1990 as DNA vaccines can induce both humoral and cell-mediated immunity. Since then, immunization by direct injection of DNA has been working out towards the induction of protective antiviral immunity in the animal models (Ulmer *et al.*, 1993; Yokoyama *et al.*, 1995; Armas *et al.*, 1996; Sakaguchi *et al.*, 1996; Chow *et al.*, 1997).

Direct injection of DNA into animals can result in immune responses to protein encoded by DNA. Although this vaccination strategy is relatively new, it has already been shown to be successful in generating protective immunity against influenza virus (Ulmer *et al.*, 1993; Larsen *et al.*, 1998), rotavirus (Herrmann *et al.*, 1996), lymphocytic choriomeningitis virus (Pedroza *et al.*, 1995; Yokoyama *et al.*, 1995), herpes simplex virus (Manickan *et al.*, 1995; Bourne *et al.*, 1996; Mangala *et al.*, 1998) and hepatitis B virus (Triyatni *et al.*, 1998). Like live-virus vaccines, DNA vaccines can induce both antibody and T-cell responses. The latter comprises both major histocompatability complex (MHC) class II-restricted $CD4^+$ T cells, most often of helper phenotype and MHC class I-restricted $CD8^+$ cytotoxic T-lymphocytes (CTLs), which kill or lyse infected cells by means of the action of protein perforin released by $CD8^+$ CTLs (reviewed in Griffiths and Tschopp, 1995). $CD8^+$ CTLs have been demonstrated to be a major host defence mechanism against many viruses since the last two decades (Yap *et al.*, 1978; Buchmeier *et al.*, 1980; Lin and Askonas, 1981; Kast *et al.*, 1986). However, the importance of CTL response induced by DNA vaccine was only highlighted recently (Doe *et al.*, 1996; Iwasaki *et al.*, 1997a; Ulmer *et al.*, 1998). At present, DNA-based immunization has yet been applied to human commercially, however, clinical trials on human have been underway (Finn, 1998; Wang *et al.*, 1998). There are many potential problems related to the use of DNA vaccines. For example, the expression of vector derived antigens may negatively influence the specific immune response in the host; the possibility of insertional mutagenesis that occur may lead to the unpredictable side effects such as switching on or off of oncogenes and tumor suppressor genes (reviewed in McDonnell and Askari, 1996). Therefore, the safety of using DNA vaccine remains an issue. Still, DNA vaccination is likely to become a valuable tool for diverse types of therapeutic interventions.

Significance of the Study

In the present study, we intended to develop DNA vaccines against the Malaysian heat resistant viscerotropic-velogenic Newcastle disease virus (NDV) strain AF2240 with both the fusion (F) and heamagglutinin-neuraminidase (HN)