UNIVERSITI PUTRA MALAYSIA

GENERATION OF A PANEL OF MONOCLONAL ANTIBODIES AGAINST THE HAEMAGGLUTININ-NEURAMINIDASE GLYCOPROTEIN OF NEWCASTLE DISEASE VIRUS STRAIN AF2240

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By

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Dedicated to Dad, Mom and Christine

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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The Malaysian velogenic-viscerotropic Newcastle disease virus (NDV) strain AF2240 is responsible for high mortality and morbidity. Monoclonal antibodies (mAbs) have been known to be useful in the identification of NDV due to their binding specificity, their homogeneity and their ability to be produced in unlimited quantities. It is, however, very difficult to obtain mAbs which are specific to NDV commercially. Therefore, this project is to develop mAbs against the local NDV strain AF2240. This velogenic-viscerotropic viral strain is a reference strain that has often been used for vaccine development.

Hybridoma cells were created by fusing NDV-hyperimmunised Balb/c splenocytes with Sp2/0-Ag14 (Sp2) myeloma cells using polyethylene glycol with the molecular weight of 1450 (PEG 1450). Positive clones were screened by ELISA. High titre producing clones were selected from a series of limiting dilutions. MAbs from stable hybridomas were further characterised by western

blot analysis, haemagglutination-inhibition test (HI) and haemolysis-inhibition test (HLI).

Eight hybridoma cell lines producing mAbs against the haemagglutinin-neuraminidase (HN) glycoprotein of NDV strain AF2240 were generated. Isotyping showed that mAbs 1B9, 2D6 and 9D7 were IgG1, mAbs 1D5, 5A10 and 5F10 were IgG2a and mAbs 2G3 and 5E10 were IgG3. Kappa (κ) light chains were found in all mAbs. They can be divided into two groups: (1) mAbs 1D5, 5A10 and 5E10 which recognised conformational and linearised epitopes and (2) mAbs 1B9, 2D6 and 9D7 which recognised only conformational epitopes. All mAbs showed positive results in the HI test but not HLI test conforming that they were specific to the HN protein and not the fusion (F) protein.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Master Sains

PENGENERASIAN SATU PANEL ANTIBODI MONOKLONAL TERHADAP GLIKOPROTEIN HEMAGGLUTININ-NEURAMINIDASE VIRUS PENYAKIT NEWCASTLE STRAIN AF2240

Oleh

LEE LIN KIAT

September 2005

Pengerusi: **Profesor Datin Khatijah Mohd. Yusoff, PhD**

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Virus penyakit sampar ayam atau virus penyakit Newcastle (NDV) strain tempatan AF2240 menyebabkan kematian yang tinggi. Antibodi monoklonal (mAb) sudah diketahui dengan kegunaannya dalam pengenalian NDV berasaskan specifikasi ikatan, keseragaman dan keupayaan dihasilkan dalam jumlah yang banyak. Namun demikian, mAb susah diperolehi secara komersil. Maka, projek ini bertujuan menghasilkan mAb terhadap NDV tempatan. Virus strain velogenic-viscerotropic ini menjadi strain rujukan yang kerap digunakan dalam penghasilan vaksin.

Sel hibridoma telah dihasilkan melalui pergabungan sel limfosit Balb/c yang telah dipertingkatkan immunisasinya terhadap NDV dengan sel miloma Sp2/0-Ag14 (Sp2) menggunakan polietilen gliko dengan berat molekul 1450 (PEG 1450). Klon positif diasai daripada teknik ELISA. Klon yang menghasilkan titer tinggi akan dipilih daripada satu siri pencairan terhad. MAb daripada hibridoma

yang stabil akan dicirikan secara lanjutan melalui analisis 'western blot', ujian penghalangan hemagglutinin (HI) dan ujian penghalangan hemolisis (HLI).

Lapan rangkaian sel hibridoma yang menghasilkan mAb terhadap NDV strain AF2240 glikoprotein hemagglutinin-neuraminidase (HN) telah digenerasikan. Pengkelasan antibodi telah menunjukkan mAb 1B9, 2D6 dan 9D7 dari sub-kelas IgG1, mAb 1D5, 5A10 dan 5F10 dari sub-kelas IgG2a dan mAb 2G3 dan 5E10 dari sub-kelas IgG3. Semua mAb mempunyai rantai ringan jenis kappa (κ). Ia juga boleh dibahagikan kepada dua kumpulan: (1) mAb 1D5, 5A10 dan 5E10 yang mengenali epitop konformasi and linear serta (2) mAb 1B9, 2D6 dan 9D7 yang mengenali epitop konformasi sahaja. Semua mAb menunjukkan keputusan positif terhadap ujian HI dan sebaliknya untuk ujian HLI bagi mengesahkan mAb adalah specifik terhadap protein HN dan bukan protein fusion (F).

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ABTS	2, 2'-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium
ADCC	antibody-dependent cell-mediated cytotoxicity
AEV	avian encephalomyelitis virus
AIV	avian influenza virus
AP	alkaline phosphatase
APS	ammonium persulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BSA	bovine serum albumin
°C	Celsius
CAV	chicken anaemia virus
CD	cluster of differentiation
cm	centimetre
CMV	cytomegalovirus
CO_2	carbon dioxide
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dTMP	deoxythymidine monophosphate
EBV	Epstein-Barr virus
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	fusion (glycoprotein)

FACS	fluorescence activated cell sorter
F_c	fragment crystallisable (Ig)
FCS	foetal calf serum
FPV	fowl pox virus
g	force of gravity
g	gram
gp120	glycoprotein 120 (HIV)
h	hour
H_2O_2	hydrogen peroxide
HA	haemagglutination
HAT	hypoxanthine, aminopterin and thymidine
HAU	haemagglutination unit
HBV	hepatitis B virus
HCV	hepatitis C virus
HFCS	hybridoma fusion and cloning supplement
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HI	haemagglutination-inhibition
HIU	haemagglutination-inhibition unit
HIV	human immunodeficiency virus
HLI	haemolysis-inhibition
HN	haemagglutinin-neuraminidase (glycoprotein)
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSV-1	herpes simplex virus -1
HSV-2	herpes simplex virus -2

HT	hypoxanthine and thymine
IBDV	infectious bursal disease virus
IBND	bivalent vaccines of IBV and NDV
IBV	infectious bronchitis virus
ICPI	intracerebral pathogenicity index
Ig	immunoglobulin
ILTV	infectious laryngotracheitis virus
im	intramuscular
IMP	inosine monophosphate
ip	intraperitoneal
iv	intravenous
IVPI	intravenous pathogenicity index
kb	kilobase
L	large (protein)
µg	microgram
µL	microlitre
µm	micrometre
M	matrix (protein)
M	molar
mA	milliampere
mAb	monoclonal antibody
mAbs	monoclonal antibodies
mg	milligram
min	minute (time)
mL	millilitre

mm	millimetre
mM	millimolar
MOPC	mineral oil plasmacytoma
NA	neuraminidase
NBT	nitro-blue tetrazolium chloride
ND	Newcastle disease
NDV	Newcastle disease virus
NEAA	non-essential amino acids
NI	neutralisation index
nm	nanometre
nM	nanomolar
NP	nucleoprotein
NT	neutralisation titre
NTE	natrium Tris EDTA
P	phosphoprotein
pAbs	polyclonal antibodies
PBS	phosphate buffered saline
PBST	phosphate buffered saline – Tween-20
PEG	polyethylene glycol
pg	picogram
pNPP	p-nitrophenyl phosphate disodium
RBC	red blood cell
RNA	ribonucleic acid
rpm	revolutions per minute
RSV	respiratory syncytial virus

RT	room temperature
RT-PCR	reverse transcription-polymerase chain reaction
s	second (time)
sc	subcutaneous
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TEMED	N, N, N', N'-tetramethylethylenediamine
TK	thymidine kinase
Tris	Tris-(hydroxymethyl)-aminomethane
UV	ultraviolet
V	volt
v/v	volume for volume
w/v	weight for volume

CHAPTER 1

INTRODUCTION

Newcastle disease (ND) is regarded throughout the world as one of the two most important diseases of poultry and other birds, the other disease being the highly pathogenic avian influenza. Its etiologic agent, the Newcastle disease virus (NDV), is a member of the family *Paramyxoviridae* and has been assigned to the genus *Avulavirus* in the subfamily *Paramyxovirinae* (Mayo, 2002; Peeters and Koch, 2002). Alexander (1989) had grouped NDV into five pathotypes based on their pathogenic signs: (1) viscerotropic velogenic NDV which causes hemorrhagic lesions in the gut; (2) neurotropic velogenic NDV shows respiratory and neurological signs but no gut lesions; (3) mesogenic NDV produces low mortality with acute respiratory disease and nervous signs in some birds; (4) lentogenic NDV shows mild and in apparent respiratory infections and (5) asymptomatic enteric NDV, avirulent viruses that appear to replicate primarily in the intestinal tract. Regardless of outbreaks or farms under constant surveillance, lack of obvious clinical signs or field experts will require confirmatory diagnosis for further identification and characterisation of the virus.

Diagnosis of ND started from the conventional techniques including virus isolation; *in vivo* estimation of pathogenicity through intracerebral pathogenicity index (ICPI) and intravenous pathogenicity index (IVPI) in one day old chicks and six weeks old chickens, respectively (Alexander, 1988); *in vitro* studies on the fusion protein cleavage site (Aldous and Alexander, 2001) and serological

tests like haemagglutination (HA) test and haemagglutination-inhibition (HI) test. These conventional methods are perceived as slow, laborious and required *in vivo* techniques. Since NDV has a 15.19 kb single-stranded negative RNA genome, reverse transcription-polymerase chain reaction (RT-PCR) was used to amplify the specific gene region using universal primers, and pathotype-specific primers or nested PCR (Aldous and Alexander, 2001). Real-time PCR that can detect minute amount (10 pg) of DNA (Tan *et al.*, 2004) and biopanning with a fusion phage that carried specific amino acid sequence to interact with surface glycoproteins (Ramanujam *et al.*, 2004) have also been performed.

Besides molecular-based techniques, monoclonal antibodies (mAbs) were intensively used in identification and differentiation of NDV strains [Iorio and Bratt, 1983 (Australia-Victoria strain); Nishikawa *et al.*, 1983 (D_{26}); Russell and Alexander, 1983 (Ulster 2C); Ishida *et al.*, 1985 (Miyadera and Taka); Abenes *et al.*, 1986 (Sato); Erdei *et al.*, 1987 (La Sota); Yusoff *et al.*, 1988 (Beaudette C); Jestin *et al.*, 1989 (Ploufragan); Panshin *et al.*, 1999 (Israel)]. MAbs were employed to study the antigenic differentiation among strains where single amino acid changes at the directed epitope can be detected (Chambers *et al.*, 1988; Yusoff *et al.*, 1989). The mAb era began in 1975 with a report in Nature by Kohler and Milstein entitled 'Continuous cultures of fused cells secreting antibody of predefined specificity'. It was reported that they could fuse immortalized myeloma cells with splenocytes which secreted a specific antibody of interest. The cell line that produces such antibodies is termed hybridoma. Since then, mAbs have become increasingly valuable in both research and therapeutic applications. The usefulness of mAbs can be characterised into three

main points: their specificity of binding, their homogeneity and their ability to be produced in unlimited quantities (Harlow and Lane, 1988). Since the antibodies produced are from one specific hybridoma cell, their identical properties make them very powerful in their ability to detect any specific epitope. Alexander *et al.* (1997) had allocated over 1500 NDV into different groups based on their ability to react with different panels of mAbs. MAbs have also been used to distinguish vaccine viruses from epizootic viruses in a given area (Srinivasappa *et al.*, 1986). Nevertheless, it is very difficult to obtain mAbs which are specific to NDV commercially. Therefore, the objectives of this study are:

a) to generate a panel of murine monoclonal antibodies against NDV strain AF2240 and

b) to characterise the selected hybridoma clones.

CHAPTER 2

LITERATURE REVIEW

Kohler and Milstein (1975) had successfully developed a technique that allows the growth of cells secreting antibodies with a defined specificity. In this technique the splenocytes isolated from an immunised animal, is fused with myeloma cells, a type of tumour cell. These hybrid cells or better known as hybridomas can be cultured *in vitro*. Antibodies secreted from hybridomas are known as monoclonal antibodies (mAbs).

2.1 Basic Concepts in Hybridoma Technology

2.1.1 Somatic Cell Hybridisation

The techniques of somatic cell fusion used by Kohler and Milstein (1975) to generate hybridomas secreting anti-sheep red blood cell with inactivated Sendai virus was a breakthrough in the field of cell biology. Cell fusion between lymphocytes and myelomas was associated with the presence of Sendai virus fusion glycoprotein, making it possible for their membrane to coalesce, cytoplasm to intermingle and multinucleated homokaryons and heterokaryons were formed (Gordon, 1975). Thus, different viruses with surface glycoproteins such as Semliki Forest virus, vesicular stomatitis virus, fowl plaque virus and influenza virus (White *et al.*, 1980, 1981) were applied to study cell fusion mechanisms. Another breakthrough was using Epstein-Barr virus (EBV) to fuse

human peripheral blood lymphocytes with human plasmacytomas to generate human mAbs (Roder, 1986).

The tumour caused by malignantly transformed antibody secreting cells is known as myeloma or plasmacytoma. According to Goding (1996), pathologists may make a morphological distinction between these terms but they may be regarded as biologically identical. Mineral oil or pristane were found to be potent inducers of myeloma in Balb/c mouse. Myelomas that were isolated using this approach were termed mineral oil plasmacytoma (MOPC) (Potter and Boyce, 1962; Potter, 1972). Myelomas that are used as fusion partners should not produce antibodies to avoid production of hybridomas that secrete more than one type of antibody. Harlow and Lane (1988) have recommended the following cell lines as good fusion partners and were successfully being used in the cited articles: FO (Davis *et al.*, 1982; Mao and France, 1984); FOX-NY (Lane, 1985); NS1/1-Ag4-1 (Nishikawa *et al.*, 1983; Russell and Alexander, 1983; Yusoff *et al.*, 1988; Llames *et al.*, 2000); Sp2/0-Ag14 (Iorio and Bratt, 1983; Long *et al.*, 1986; Letellier *et al.*, 2001; Fontes *et al.*, 2005) and X63Ag8.653 (Davis *et al.*, 1982; Srinivasappa *et al.*, 1986).

Splenocytes are obtained from the immunised mouse through splenectomy. A spleen from an immunised mouse contains approximately 5×10^7 to 2×10^8 splenocytes (Harlow and Lane, 1988). Marusich (1988) reported that 1×10^5 splenocytes were sufficient to generate hybridomas. Hybridoma cells are created by fusing splenocytes from an immunised animal with myeloma cells to enable the hybridomas to possess both the antibody secreting properties of the parent