UNIVERSITI PUTRA MALAYSIA

MAPPING OF NUCLEOCAPSID PROTEIN (NP) EPITOPES AND NP-PHOSPHOPROTEIN (P) INTERACTIVE DOMAINS OF NEWCASTLE DISEASE VIRUS WITH NP MONOCLONAL ANTIBODIES

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Doctor of Philosophy

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Chairman : Professor Datin Khatijah Mohd. Yusoff, PhD

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The Newcastle disease virus (NDV) is an economically important poultry virus which replicates in certain human cancer cells. This virus contains a negative single stranded RNA genome which encodes for the nucleocapsid protein (NP), phosphorylated nucleocapsid-associated protein, phosphoprotein (P); matrix protein (M); fusion protein (F); haemaglutinin-neuraminidase protein (HN) and the RNA-directed RNA polymerase, large protein (L).

The NP is the most abundant protein found in NDV. In this study, a panel of monoclonal antibodies (mAbs) against NP was developed to study the NP-P interactions in NDV. The spleen cells of Balb/C mice immunized with purified NP obtained from the velogenic NDV strain AF2240 were fused with myeloma cells (Sp2/0-Ag14 cell line). A panel of six mAbs were produced and characterized. Four of the mAbs secreted immunoglobulins from class IgG2a with kappa light chains and the remaining two were from class IgG1 with kappa light chains. Cross-reactivity test against the NPs from other six NDV strains showed that mAbs, a2, a2s and b2 cross-reacted with all NDV strains, while mAb b3 showed specificity towards the NP of

strain AF2240, the strain that was used earlier to immunize the mice. The other two mAbs, b4s and c1, demonstrated cross-reactivity amongst the various viral strains with varying reactivities. These results indicate that certain epitopes recognized by the mAbs were well conserved in all NDV strains whilst the other epitopes may have undergone some structural changes.

The antigenic sites of NP bound by the mAbs were localized by Western blot analysis. Four C- and N-terminally truncated NP mutants were purified from *Escherichia coli*, blotted to the nitrocellulose membrane and probed with NP mAbs. The results show that the antigenic sites bound by mAbs a2, a2s and b2 were located within amino acids 441 to 489 of the C-terminal of NP. On the other hand, antigenic sites that were recognized by the mAbs, b3 and b4s were located on the N-terminal half of NP from 26 to 121 amino acid residues. MAb c1 bound to all C- and N-truncated mutants indicating that the antigenic sites recognized by mAb c1 may be located within amino acids 122 to 375.

One of the mAb, a2s was further used as a tool in protein-protein interaction study between assembled NP (NP_{NC}) and P. The NP_{NC} was purified from *E. coli* by ammonium sulphate precipitation and sucrose garadient. In determining the interaction regions of P that bind to NP_{NC}, the mAb is used in immunoprecipitating the radioactively labeled Ps-NP_{NC} complex. The failure of certain P deleted mutants to form complex with NP_{NC} demonstrated that the regions of P which were deleted from those mutants were responsible with the binding to NP_{NC}. After 18 different N- and C-terminally truncated P mutants were tested in the radioimmunoprecipitation assay, it showed that the region of P that binds to NP_{NC} is located within the internal region of

C-terminal half of P, from amino acids 243 to279. In agreement with the radioimmunoprecipitation results, protein binding assay, another assay that was carried out to determine the P-NP_{NC} interactive domain also showed that the interactive domain was mapped to the internal region of the C-terminal half of P (amino acids 224-279). A slightly bigger region of interaction domain was determined by the latter assay compared to the former assay was due to its nature and higher sensitivity of the assay. Nevertheless, both assays showed that the N-terminal half and immediate C-terminal end of P is not involved in the binding of P to NP_{NC}.

To further explore the interactions between P and NP, Far Western blotting was carried out to determine the binding domain of P to NP monomer, NP_O. The NP_O was obtained by fractionating the NP_{NC} in SDS-PAGE. In this assay, the same deleted mutants that are utilized in mapping P-NP_{NC} interactive domain were also used. The assay showed that amino acids 224-279 were indispensable for the P-NP_O binding. Interestingly, these were the same amino acids that were responsible for the P-NP_{NC} interaction. These results indicate that these amino acids were crucial for interaction between P and NP and may play bigger roles in transcription and replication of viral genome.

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

PEMETAAN EPITOP PROTEIN NUKLEOKAPSID (NP) DAN DOMAIN INTERAKTIF NP-FOSFOPROTEIN VIRUS PENYAKIT NEWCASTLE MENGGUNAKAN ANTIBODI MONOKLONAL NP

Oleh

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Oktober 2006

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Virus penyakit Newcastle (NDV) adalah virus yang menyebabkan kerugian dalam industri ternakan ayam dan juga boleh bereplikasi dalam sel kanser manusia tertentu. Ia mengandungi genom bebenang tunggal (negatif) RNA yang mengkodkan protein nukleokapsid (NP), protein fosforilasi yang bertuapan dengan nukleokapsid, fosfoprotein (P), protein matriks (M), protein tuapan (F), protein hemaglutinin-neuraminidas (HN) dan polimeras RNA, protein besar (L).

NP merupakan protein yang boleh didapati dengan banyak dalam NDV. Dalam kajian ini, satu panel monoclonal antibodi (mAb) terhadap NP telah dihasilkan untuk digunakan dalam kajian interaksi NP-P NDV. Sel limpa mencit Balb/C yang diimunisasi dengan NP yang diperolehi daripada NDV veliogenik strain AF 2240 telah digabungkan dengan sel miloma (sel Sp2/0-Ag14). Satu panel yang terdiri daripada enam mAb telah dihasilkan dan dicirikan. Empat daripada mAb tersebut merembeskan immunoglobulin daripada kelas IgG2 dengan rantai ringan kappa dan dua lagi daripada kelas IgG1 dengan rantai ringan kappa. Ujian reaksi silang terhadap

NP daripada enam strain NDV lain menunjukkan mAbs a2, a2s dan b2 bereaksi silang dengan semua strain NDV, manakala mAb b3 menunjukkan spesifisiti terhadap NP strain AF2240 iaitu strain sama yang digunakan untuk imunisasi mencit. Dua lagi mAb iaitu b4s dan c1 menunjukkan reaksi silang secara variasi terhadap semua strain tersebut. Keputusan ini menunjukkan terdapat epitop tertentu yang dikenalpasti oleh mAb adalah terpelihara dalam semua strain NDV manakala epitop lain telah mengalami perubahan struktur.

Tapak antigenik NP yang dikenalpasti oleh mAb telah diidentifikasi dengan analisis blot Western. Empat mutan NP yang terpotong pada terminal-C and -N yang diekstrak daripada *Escherichia coli* diblot kepada membran nitroselulosa dan diprob dengan mAb NP. Keputusan menunjukkan tapak antigenik yang dikenalpasti oleh mAb a2, a2s dan b2 terletak pada asid amino 441 hingga 489 terminal-C NP. Manakala tapak antigenik yang dikenalpasti oleh mAb b3 dan b4s terletak pada terminal-N NP dari asid amino 26 hingga 121. MAb c1 melekat pada semua mutan NP yang terpotong pada terminal-C and –N dan ini menunjukkan tapak antigenik yang dikenalpasti oleh mAb c1 terletak pada asid amino 122 hingga 375.

Satu daripada mAb iaitu a2s telah digunakan dalam kajian interaksi antara NP yang berhimpun, NP_{NC} dan P. NP_{NC} diekstrak daripada *E. coli* dengan kaedah presipitasi ammonium sulphate and gradien sukros. Dalam menentukan domain interaksi P yang bergabung kepada NP_{NC}, mAb tersebut digunakan untuk immunopresipitat pelbagai kompleks mutan P-NP_{NC} yang dilabel dengan radioaktif. Selepas 18 mutan P yang terpotong pada terminal-C and –N diuji dengan asai radioimmunopresipitasi, keputusan menunjukkan domain P yang bergabung dengan NP_{NC} adalah terletak

dalam bahagian dalaman terminal-C iaitu daripada asid amino 243 sehinnga 279. Asai gabung protein juga meunjukkan domain P yang bergabung kepada NP_{NC} adalah terletak pada bahagian yang sama (asid amino 224-279). Domain interaksi yang diidentifikasi oleh asai gabung protein adalah lebih besar daripada asai radioimunopresipitasi adalah disebabkan bentuk tafsiran asai gabung protein yang lebih sensitif berbanding asai imunopresipitasi. Walau bagaimanapun, kedua-dua asai menunjukkan terminal-N dan hujung terminal-C P tidak terlibat dalam interaksi P kepada NP_{NC}.

Untuk mendalami kajian interaksi P dan NP, blot Western Far telah digunakan dalam mengenalpasti domain P yang bergabung dengan monomer NP, NP_O. NP_O diperolehi dengan fraksinasi NP_{NC} di dalam SDS-PAGE. Dalam asai ini, mutan P yang sama yang telah digunakan dalam kajian interaksi P-NP_{NC} telah digunakan. Asai tersebut menunjukkan asid amino 224 sehingga 279 adalah terlibat dalam interaksi P-NP_O. Yang menariknya, asid amino tersebut juga terlibat dalam interaksi P-NP_{NC}. Ini menunjukkan asid amino tersebut sangat penting dalam interaksi P-NP dan kemungkinan memainkan peranan lebih besar dalam transkripsi dan replikasi genom virus.

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I certify that an Examination Committee met on 4th October 2005 to conduct the final examination of Raha Ahmad Raus on her Doctor of Philosophy thesis entitled "Mapping of Nucleocapsid Protein (NP) Epitopes and NP-Phosphoprotein (P) Interactive Domains of Newcastle Disease Virus with NP Monoclonal Antibodies" in accordance with Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degraee. Members of the Examination Committee are as follows:

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Date: 16 JANUARY 2007

DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

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RAHA AHMAD RAUS

Date: 11 NOVEMBER 2006

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ABTS	2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic)
ATP	adenosine triphosphate
BCIP	bromochloroindoyl phosphate
bp	base pair
BSA	bovine serum albumin
C	constant domain
^{o}C	degree Celcius
cDNA	complementary deoxyribonucleic acid
CDR	complementarity-determining region
CE	conformational epitope
CITE	cap-independent translation enhancer
cpm	count per minute
DEA	diethanolamine
dH_2O	distilled water
DMEM	Dulbeccos's modified Eagle's medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DTT	1,4-dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
F	fusion protein
Fab	fragment antigen binding

FACS	fluorescence-activated cell sorter
Fc	crstallizable fragment
FBS	fetal bovine serum
Fv	variable fragment
H	heavy chain
h	hour
HAMA	human anti-mouse antibodies
HAT	hypoxanthine, aminopterin and thymidine media
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HN	haemaglutinin-neuraminidae protein
hPIV3	human parainfluenza virus type 3
HT	hypoxanthine and thymidine media
hRSV	human respiratory syncytial virus
Ig	immunoglobulin
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kiloDalton
L	large protein (polymerase)
LB	Luria-Bertani
LE	linear epitope
LPMV	La Piedad Michoacan Mexico virus
M	matrix protein
mA	milliampere
mAb	monoclonal antibody
MCS	multiple cloning site
mg	milligram

min	minute
ml	milliliter
mM	millimolar
mRNA	messenger ribonucleic acid
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar
NBT	nitro-blue tetrazolium chloride
NDV	Newcastle disease virus
nm	nanometer
NP	nucleocapsid protein
NP_O	nucleocapsid monomer
NP_{NC}	assembled nucleocapsid
NP-40	nonidet P-40
OD	optical density
P	phosphoprotein
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline containing Tween 20
PCR	polymerase chain reaction
PEG	polyethylene glycol
pNPP	p-nitropheyl phosphate disodium
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
RSV	respiratory syncytial virus

RT	room temperature
S	second
SB	standard binding buffer
scFv	single-chain variable fragment
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
TAE	tris-acetate EDTA buffer
TBS	tris buffered saline
TBS-T	tris buffered saline containing Tween 20
V	variable domain
V_H	variable domain of heavy chain
V_L	variable domain of light chain
VSV	vesicular stomatitis virus
v/v	volume for volume
w/v	weight for volume

CHAPTER 1

INTRODUCTION

One of the immune responses to eliminate pathogens and other foreign molecules from our body is by secreting antibodies that act specifically against them. Antibodies are produced in large quantities by plasma cells which are developed from precursor B cells. However, Kohler and Milstein (1975) successfully produced the antibodies *in vitro* through permanent tissue culture cell lines. These antibodies are produced by hybridoma cells which are generated from a fusion of immunized B-cells and myeloma tumor cells. These antibodies are considered monoclonal (mAb) as they are secreted from a single cell or clone. Therefore these antibodies are specific against a particular epitope of an antigen. Although initially, these mAbs which were produced had no practical application, further investigations by other scientists have resulted in many beneficial discoveries. At present, the production of mAbs has resulted in the generation of an industry with an economic impact measured in billions of dollars.

MAbs with specificity to tumour-associated antigens have been applied in various diagnostic assays *in vitro* and as well as *in vivo* by using gamma-emitting radio-labelled mAbs to identify the location of tumor in patient. It has also been used to treat cancerous and non-cancerous diseases and act as a carrier to various toxin, cytostatic drug and radionuclides to treat diseases (Govindan *et al.*, 2005). Poisoning with digoxin and other potent, low formula mass poisons, such as colchicine and tricyclic antidepressants could be successfully treated using specific mAbs fragment, Fab (Flanagan and Jones, 2004). Identification of bacteria, virus and protozoa in

clinical samples are easily conducted by using mAbs that are specific against each microbe. For certain bacterial and viral infections, the use of neutralizing mAbs confers impressive protection against the pathogen (Mc Cann *et al*., 2005).

Other than its obvious contribution in clinical medicine, the use of mAbs has a strong impact towards the knowledge of biological sciences. For example, in neuroscience research, mAbs provide purified membrane associated proteins such as pumps, channels, receptors and cell-adhesion molecules so that their structures could be studied at high resolution. MAbs to cytoskeletal proteins, organelles, and protein kinases have revealed that specific molecules are concentrated in anatomically distinct regions of the cell and protein kinase has been shown to be a major postsynaptic constituent in many synapses. The antibodies are also used to identify functionally related subpopulations of neurons and describing neural cell lineages (Valentino *et al*., 1985).

In the present study, mAbs are produced using the hybridoma technology. These were then characterized and, used to map the NP antigenic sites and to study protein-protein interactions in Newcastle disease virus (NDV). This particular virus infects many avian species. In many countries worldwide, NDV infections result in great economic loses amounting billions of dollars as infections of chicken with velogenic strain of NDV causes up to 100% mortality. NDV contains a negative single stranded RNA genome which encodes at least six structural proteins, the nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN) and large polymerase (L) proteins. Among these proteins, the NP, P and L proteins are involved in viral transcription and replication. The interaction between P and NP is