

**Development and Characterization of Murine
Monoclonal Antibodies Capable of Neutralizing
Vaccinia Virus**

By

Ran Chen

**A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of**

MASTER OF SCIENCE

**Department of Medical Microbiology and Infectious Diseases
University of Manitoba
Winnipeg, Manitoba
Canada**

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ACKNOWLEDGEMENTS

I would first like to thank my supervisor, Dr. Magdy Dawood, for offering me the opportunity to work in his laboratory. Without him, this thesis would not have been possible. I thank him for his patience and encouragement that carried me on through difficult times, and for his insights and suggestions that helped to shape my research skills. He offered me every chance to do experiments that were interesting to me. He was always available for questions and discussions with great patience. As a result of experiences in his laboratory, I feel ready to tackle whatever scientific pursuits come next.

I would also like to thank my supervisory committee members including Dr. John Wilkins, Dr. Jody Berry and Dr. Kevin Coombs for their support and guidance during my Master career. Each person on my committee contributed reagents, time, and expertise for my project.

I wish to extend my gratitude to Dr. Paul Hazelton and Ms. Petty Sauder for their time, assistance, and guidance with the electron microscopy and hybridoma development work respectively. Their invaluable contributions are very much appreciated.

The people from the Cadham Provincial Laboratory made the day-to-day experience of work worthwhile and enjoyable. In particular, I would like to thank Dr. Paul Van Caesele and Mr. Gerry Smart for their attention and great advice.

To acknowledge former members of the laboratory, I would like to thank Ms. Rhianon Harris and Ms. Teresa Takla. They turned to be my good friends and helped me out through the hardest period of my project. I enjoyed all the vivid discussions we had on various topics and had lots of fun being a member of this fantastic group.

In addition, I acknowledge the Department of Medical Microbiology support staff including Dr. Xi Yang for helping me out on financial problems and Ms. Angela Nelson for always making sure that deadlines and requirements were met and for easing all of the technical and financial concerns of this process.

Finally, I give enormous thanks to my family, especially my husband, Zaikun Xu, and my parents. I am extremely blessed in their support and love, which they gave time and time again when my hopes and enthusiasm were low. My husband was there for me with open ears and heart, always wanting to hear about my day, and ready to cheer me up whenever necessary. I thank him for his faith and love.

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ABBREVIATIONS

AP	Alkaline Phosphatase
BEA	2-bromoethylamine
BEI	Binary Ethyleneimine
BPL	Beta-propiolactone
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CEF	Chicken Embryo Fibroblast
CEV	Cell-associated Enveloped Virus
CMV	Cytomegalovirus
CPE	Cytopathic Effect
DMEM	Dulbecco's Modified Eagle's Medium
DOD	Department of Defense
EBV	Epstein-Barr Virus
ECL	Enhanced Chemiluminescent
EEV	Extracellular Enveloped Virus
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Electron Microscope
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
HCL	Hybridoma Cell Line
HAT	Hypoxanthine-Aminopterin-Thymidine
hMAb	Human Monoclonal Antibody
IEV	Intracellular Enveloped Virus
Ig	Immunoglobulin
IMV	Intracellular Mature Virus
IND	Investigational New Drug
IP	Intraperitoneal

IV	Immature Virus
kDa	Kilodalton
L-variant	Large-variant
MAb	Monoclonal Antibody
MOI	Multiplicity of Infection
MT	Microtubules
MVA	Modified Vaccinia virus Ankara
MWCO	Molecular Weight Cut Off
MYCO	Mycomycin
NYCBH	New York City Board of Health
ORF	Open Reading Frame
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PENGEN	Penicillin and Gentamicin
PEG	Polyethylene Glycol
PFU	Plaque Forming Unit
PRA	Plaque Reduction Assay
RSV	Respiratory Syncytial Virus
SC	Subcutaneous
SCR	Short Consensus Repeats
S.D.	Standard Deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TGN	<i>trans</i> -Golgi Network
Vero cell	Africa Green Monkey cell
VIG	Vaccinia Immune Globulin
VV	Vaccinia Virus
WB	Western Blot assay
p.i.	post infection
WR	Western Reserve

ABSTRACT

INTRODUCTION: Since the eradication of smallpox in 1977, mass vaccination efforts against it have been discontinued. Thus, the majority of the younger population is susceptible to both smallpox virus and vaccinia virus (VV). The re-emergence or intentional release of smallpox will present a serious threat to global health. There are limited supplies of smallpox vaccine, which is associated with significant complications, and pooled anti-VV human immune globulin (VIG) that can be used as prophylaxis or to treat smallpox-exposed individuals. We are developing murine monoclonal antibodies (MAbs) able to neutralize VV. The developed MAbs may be useful in establishing a rapid diagnostic test for the detection of VV infection or providing the genetic materials needed for developing recombinant antibodies suitable for human use.

METHODS: VV Western Reserve (WR) strain was propagated in HeLa or Chicken Embryo Fibroblast (CEF) cell lines, purified through a 36% sucrose cushion and inactivated by binary ethyleneimine (BEI). Female BABL/c mice were immunized with inactivated VV. Hybridoma cell lines (HCLs) were developed from spleen cells of the mice with high neutralizing antibody titers. Tissue culture supernatants from the developed HCLs were screened by Enzyme-Linked Immunosorbent Assay (ELISA) and Plaque Reduction Assay (PRA) for their abilities to produce neutralizing antibodies against VV. HCLs producing neutralizing antibodies were sub-cloned by limiting dilution method. Highly neutralizing MAbs were isotyped and purified. The effect of using increasing microgram amounts of each MAb or mixtures of two MAbs on VV neutralization has been determined. Specific target proteins recognized by MAbs were

detected by western blot assay (WB). The abilities of the developed MABs to neutralize other three VV strains, Large-variant (L-variant), IHD-W and New York City Board of Health (NYCBH), were measured.

RESULTS: We have developed 261 HCLs producing anti-VV antibodies; 65 of them neutralized VV. Twelve HCLs were sub-cloned. We developed 79 sub-clones producing neutralizing MABs. The majority of them were immunoglobulin IgG1/ κ isotype. Four highly neutralizing MABs were concentrated and purified. They were able to neutralize 50% of VV infection at 0.01-0.1 μ g in PRAs. Synergistic effects on VV neutralization were observed when mixing two MABs from clones, 1-E9-1-E4 and 2-B7-9-E6, at the amounts giving about 20% and 40% VV neutralization. Based on the WB results, the developed MABs are recognizing 75 kilodalton (kDa), 45 kDa, 35 kDa or 8 kDa WR VV proteins. The abilities of the developed MABs to neutralize other strains of VV varied.

CONCLUSIONS: Several HCLs producing antibodies against VV were developed. Highly neutralizing MABs against WR VV have been produced and purified. Virus neutralization is dose dependent and some of MABs have synergistic neutralization effects on each other. Most of the MABs were targeting the same three virus envelope proteins indicating that these proteins contain important epitope(s) responsible for the neutralizing effects by the developed MABs. Variable neutralization abilities were observed on three other VV strains indicating their immunobiologic differences with WR VV strain. The developed MABs may be used as a research tool to study VV pathogenesis or for the development of chimeric antibodies for clinical applications.

CHAPTER 1 INTRODUCTION

1.1 Vaccinia virus

VV belongs to the genus *Orthopoxvirus* of the family *Poxviridae*. Many members within this genus, such as monkeypox, cowpox, and variola (the causative agent of smallpox) viruses, share high degree of identity in their nucleotide sequences (Fenner, *et al.* 1989; Rotz, *et al.* 2001). VV is a large and complex double-stranded DNA virus that replicates within the cell cytoplasm (Wittek, 1982; Minnigan and Moyer, 1985; Moss, 1990). It has a wide host range and an unknown origin (Baxby, 1981; Moss, 1990; Buller and Palumbo, 1991). There are many strains of VV with different biological properties. The VV 187-kilobase genome encodes 150 - 200 proteins involved in viral infection, replication and virion morphogenesis (Goebel, *et al.* 1990; Bray, 2003). Approximately 279 polypeptides are induced during virus infection (Carrasco and Bravo, 1986). “VV was the first animal virus to be seen microscopically, grown in tissue culture, accurately titered, physically purified, and chemically analyzed” (Moss, 1990).

1.2 Life cycle of vaccinia virus

There are four different forms of virus particles within VV life cycle, including intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (Smith and Law, 2004) (Figure.1.1). IMV particles, which represent the most abundant infectious form of VV, are formed within the cytoplasmic factories and mostly retained in the host cells until lysis. A fraction of IMV becomes further wrapped by a double intracellular membrane

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Figure.1.1 Cartoon of viral morphologic changes during vaccinia life cycle.

This diagram summarizes the different forms of VV particles in its life cycle. IMV are the result of viral protein processing from their precursors IV and move through microtubules (MT) to the wrapping membranes derived from the *trans*-Golgi network (TGN) or early endosomes. These particles may be further enveloped by double membranes to yield the IEV. IEV are transported to the cell surface via microtubules and fuse with the plasma membrane to form CEV. The release of CEV from cells results in formation of EEV particles. (Smith, *et al*, 2002)

derived from the early tubular endosomes or TGN to form the IEV. IEV is an intermediate form between IMV and CEV or EEV. Some IEV particles transport to the cell surface where their outermost membranes fuse with the plasma membranes exposing the enveloped virions on the cell surface. The particles remained on the cell surface are called CEV. CEV induces the formation of actin tails that drive CEV particles away from the cell forming EEV. CEV and EEV are physically identical to each other. CEV are important for cell-to-cell spread (Smith, *et al*, 2002). EEV mediate the long-range dissemination of virus in cell culture and, probably, *in vivo*. They are produced in varying amounts by different strains of VV (Payne, 1980). IMV and EEV are the two major infectious forms among the four different VV forms.

IMV and EEV are different in their membrane structures, antigenicities, and various other biological properties. They use different mechanisms to infect host cells, notably by binding to different cell surface receptors (Vanderplasschen and Smith, 1997). IMV are assumed to be the particles responsible for the spread of VV between hosts because the EEV outer membrane is too fragile to survive the physical environment outside the host, and once the outer membrane is broken, it will release the fully infectious and relatively stable IMV particles (Ichihashi and Oie, 1996). EEV represent the biologically relevant forms of the virus to spread the infection within the host *in vivo* (Payne, 1980; Blasco and Moss, 1991) and seem more selective in terms of affinity to different cells or tissues (Vanderplasschen and Smith, 1997). It has been reported that IMV and EEV penetrate host cells by membrane fusion and endocytosis respectively (Ichihashi and Oie, 1996; Vanderplasschen, *et al*, 1998a). In this model, EEV entry consists of binding to the cells, endocytosis and disruption of the EEV outer membranes within the endosomes releasing

the infectious IMV, which then bind to and fuse with the endosome membranes. Based on this model, the EEV outer membrane may work as a coat to protect the inside IMV particle against antibody neutralization as well as engage in the binding to the cell surface.

1.3 Neutralizing antibodies induced by vaccinia infection

The humoral immune response against VV infection is mainly composed of complement-fixing, hemagglutination-inhibiting and neutralizing antibodies activities (Henderson and Inglesby, *et al*, 1999; Harrop, *et al*, 2004). However, the first two types of antibodies are not associated with effective immunity and their titers decline significantly within about 12 month post-infection (p.i.) (Henderson and Inglesby, *et al*, 1999). The real protection seems to be associated with the presence of neutralizing antibodies, which can last for years after vaccination or infection (Rodriguez, 1985; Henderson and Inglesby, *et al*, 1999). The specificities and functional roles of the neutralizing antibodies are crucial to either understand the pathobiology of the VV, to develop effective antiviral therapy, or to develop human neutralizing MAbs for prophylaxis applications against smallpox infection.

Neutralizing antibodies induced by VV administration are genus-specific and cross-protective with other Orthopoxviruses such as monkeypox, cowpox, and variola viruses (Fenner, *et al*. 1989; Rotz, *et al*. 2001). Neutralizing antibodies can be detected 7 - 10 days after vaccination and persist in 75% of vaccinated individuals for 10 years and up to 30 years after receiving two or three doses of the vaccine (Centers for Disease Control and Prevention (CDC), <http://www.cdc.gov/nip/publications/pink/smallpox.rtf>).

In general, anti-IMV antibodies may primarily protect by neutralizing the virus particles. On the other hand, anti-EEV antibodies may primarily protect by limiting viral spread after infection. There still remains a debate as to the relative *in vivo* contributions of the neutralizing antibodies against IMV *versus* EEV forms of the virus to the control of viral infection.

Identifying key epitopes of VV recognized by the neutralizing antibodies is important for developing a clear understanding of the mechanisms of protection offered by the currently used prototypic smallpox vaccine. In addition, it is also instructive for ongoing efforts to design alternative smallpox vaccines.

1.3.1 Neutralizing antibodies against IMV

Of the two major infectious forms of VV, IMV and EEV, the most potent neutralizing antibodies are found against the IMV form (Ichihashi and Oie, 1996; Law and Smith, 2001). As mentioned previously, unlike EEV contributing to the distant virus spread, IMV are probably more efficient in local transmission by fusion to adjacent cells (Blasco and Moss, 1991; Smith and Vanderplasschen, 1998). Studies have demonstrated that antibodies against either IMV or EEV particles can be protective (discussed below). In the context of a vaccine development to prevent host-to-host transmission of the virus, antibodies against IMV surface antigen might be expected to play a major role.

Virus proteins targeted for antibody neutralization have been mapped in IMV (Gordon, *et al*, 1991; Wolffe, 1995; Hsiao, 1999) and they include five of the eleven known IMV membrane proteins. They are encoded by open reading frames (ORF): A17L, A27L, D8L,

Table 1-1 List of vaccinia membrane proteins of IMV or EEV particles related to the neutralization effects.

VV gene	particle type	protein size(kDa)	references
A13L	IMV	8	<i>Salmons, 1997; Pulford, 2004</i>
A17L	IMV	21	<i>Wallengren, 2001</i>
A27L	IMV	14	<i>Rodriguez, 1985, 1987; Lai, 1991; Demkowicz, 1992</i>
D8L	IMV	32	<i>Hsiao, 1999; Mathew, 2001</i>
H3L	IMV	35	<i>Lin, 2000</i>
L1R	IMV	25	<i>Wolffe, 1995; Ichihashi and Oie, 1996</i>
A33R	EEV	23-28	<i>Roper, 1996</i>
A34R	EEV	24-28	<i>Duncan and Smith, 1992; McIntosh and Smith, 1996</i>
A56R	EEV	85/89	<i>Payne and Norrby, 1976; Shida, 1986</i>
B5R	EEV	42	<i>Engelstad, 1992; Isaacs, 1992</i>
F13L	EEV	37	<i>Hirt, 1986; Blasco and Moss, 1991</i>

L1R and H3L (refer to Table 1-1).

1.3.1.1 A27L protein

A 14 kDa protein, a product synthesized late during virus infection, is encoded by A27L ORF (Rodriguez, 1985). It is one of the best characterized and most promising proteins to develop protective immune response against VV. It forms covalently linked-trimers and is localized in the envelope of the IMV particle (Rodriguez *et al*, 1987; Sodeik *et al*, 1995). The A27L gene is highly conserved in the evolution of the orthopoxviruses (Olson, *et al*, 2004).

The 14 kDa protein plays an important role in the biology of VV by acting in virus-to-cell and cell-to-cell fusion (Dallo *et al*, 1987; Rodriguez *et al*, 1987; Gong, *et al*, 1990; Rodriguez and Smith, 1990). A structural model of the coiled-coil self-associated trimeric structure for the wild-type A27L protein has been proposed by Vazquez *et al*. (1998). The N-terminus of the 14 kDa protein consists of a heparin-binding domain, a fusion domain and a domain responsible for interacting with proteins or lipids in the Golgi stacks required for EEV formation and virus spread (Vazquez and Esteban, 1999). Rodriguez (1993) demonstrated that the 14 kDa protein trimers can form a stable protein complex through the C-terminal domain with a 21 kDa dimeric protein (A17L gene).

Ramirez (2002) laboratory has demonstrated, in a mouse model, that administration of a MAb against the 14 kDa protein can control, to different extents, systemic poxvirus infection through inhibition of the virus replication in target tissue. They also suggested (2002) that this MAb plays its role when the majority of virus releases after cell lysis

instead of the EEVs' natural egress from the host cell. This finding provides an interesting antiviral molecule that can be used to protect against orthopoxvirus infection. Additionally, Otero (2005) indicated that administration of DNA plasmids encoding A27L gene alone or along with other VV neutralizing antibodies target genes in mice was able to induce an efficient protection against a lethal poxvirus challenge (5×10^6 PFU) given by intraperitoneal (IP) injection.

1.3.1.2 L1R protein

L1R ORF encodes a 25 kDa membrane protein localized on the inner membrane of the IMV (Franke *et al*, 1989; Ravanello, *et al*, 1993, 1994a, 1994b). It is synthesized late in infection and is a myristylated component of the IMV membrane (Franke, *et al*, 1989, 1990; Ravanello and Hruby, 1994a, 1994b; Wolffe, *et al*, 1995). Use of an inducible recombinant virus demonstrated that absence of L1R gene results in a shutdown of morphogenesis at an early stage and preventing the formation of IMV particles (Ravanello and Hruby, 1994a, 1994b). Importantly, L1R protein is another major target of VV neutralizing antibodies, in addition to the A27L protein mentioned above, suggesting that it has a role in virus entry (Wolffe, *et al*, 1995; Ichihashi and Oie, 1996). A MAb recognizing the L1R protein blocked IMV entry at the post-binding step, reflecting that the L1R protein plays a role in virus penetration (Wolffe, *et al*. 1995). Mice vaccinated with a plasmid encoding L1R gene produced IMV-neutralizing antibodies although the animals were not protected from a lethal virus challenge. However, mice immunized with DNA expressing both L1R and EEV proteins (A33R

and/or B5R proteins) were protected from such a challenge (Hooper, *et al*, 2000, 2003; Fogg, *et al*, 2004).

1.3.1.3 H3L protein

A 35 kDa polypeptide, encoded by the H3L ORF, is a VV integral membrane protein (Davies, *et al*, 2005). The H3L protein is found predominantly in the envelope of IMV (Da Fonseca, 2000a, 2000b). Soluble H3L protein binds to a cell-surface molecule, heparan sulfate, a glycosaminoglycan known to mediate the attachment of many viruses such as herpes simplex virus (WuDunn and Spear, 1989; Shieh, *et al*. 1992) and foot-and-mouth disease virus (Jackson, *et al*, 1996), and blocks the adsorption of IMV, indicating that H3L protein is a VV protein involved in binding to target cells. The H3L gene is strongly conserved between variola virus and VV. There is 96% amino acid identity between the H3L proteins derived from WR VV and variola virus, which may contribute to the cross-protection against variola virus resulted from VV vaccination (Davies, *et al*, 2005).

H3L protein is among the VV proteins most frequently recognized by sera from vaccinated people (Davies, *et al*, 2005). Human anti-H3L antibodies have demonstrated the abilities of neutralizing VV *in vitro* by Lin (2000). Additionally, immunization of mice against H3L protein induced strong neutralizing antibodies, and H3L protein-immunized animals or animals that received passive transfer of anti-H3L antibodies were protected against a lethal challenge with pathogenic VV (Davies, *et al*, 2005). These results indicated that H3L protein is a good candidate to be included in a subunit vaccine against smallpox virus.

1.3.2 Neutralizing antibodies against EEV

In animal models, the poor protection of vaccines, comprised largely of inactivated IMV, has been attributed to the absence of antibodies against EEV (Turner and Squires, 1971; Boulter and Appleyard, 1973; Davies, *et al*, 2005). Although neutralization of IMV by antibodies against IMV membrane proteins is well documented, inhibition of EEV infectivity has been more difficult to demonstrate (Ichihashi and Oie, 1996; Vanderplasschen and Smith, 1997; Law and Smith, 2001). EEV is also more resistant than IMV to inactivation by complement (Vanderplasschen, *et al*, 1998b). Galmiche (1999) also pointed out that there is a disparity between the poor *in vitro* neutralizing and good *in vivo* protective effects of antibodies to VV extracellular envelope proteins. The enhanced protection was achieved by immunization schemes that elicit antibodies against both EEV and IMV proteins (Hooper, *et al*, 2000, 2003; Fogg, *et al*, 2004) or by passive administration of combinations of antibodies to IMV and EEV proteins (Fogg, *et al*, 2004). The possible explanation for this phenomenon is that the complement may contribute to the lysis of the outer EEV membrane in the presence of antibody against EEV and releasing the IMV. As the EEV membrane no longer protects the IMV any more, the naked IMV become sensitive to neutralization by the antibodies against it (Fogg, *et al*, 2004).

At least 10 proteins, which are absent from IMV, are associated with the outer envelope of EEV (Payne, 1978; Payne and Kristenson, 1979). Six of them are known to be present in the EEV outer envelope. They are A33R encoded protein, 23 - 28 kDa glycoproteins; A34R protein, a triplet of glycoproteins; A56R protein, the virus hemagglutinin; B5R

protein, a 42 kDa glycoprotein; and F13L protein, a non-glycosylated 37 kDa protein (refer to Table 1-1). These proteins endow EEV with different biological and immunological properties (Boulter, 1969; Appleyard, *et al*, 1971; Turner and Squires, 1971; Boulter and Appleyard, 1973). Except for the hemagglutinin, interference with the expression of any of those proteins by deletion or repression was shown to have a dramatic impact on the ability of the virus to spread from cell to cell in tissue culture and to form plaques (Blasco and Moss, 1991; McIntosh and Smith, 1996; Herrera, *et al*, 1998; Mathew, *et al*, 1998). Modifications of these genes were also shown to attenuate the virus *in vivo* (Blasco and Moss, 1991; Engelstad and Smith, 1993). Although several studies have been performed on these genes, still very limited information is known about their involvement in EEV assembly and release from the infected cells, as well as their roles in attachment to the cell surface, cell entry and uncoating (Galmiche, *et al*, 1999). Since we are studying the IMV VV particles in this study, I will only mention the B5R protein, which is considered as one of the most important surface proteins on EEV, as an example.

1.3.2.1 B5R protein

B5R ORF encoded protein is a 42 kDa outer envelope glycoprotein (Isaacs, *et al*, 1992). It is one of many EEV-specific proteins that are vital for the optimal formation of EEV (Engelstad *et al*, 1992; Isaacs, *et al*, 1992; Engelstad and Smith, 1993; Wolffe, *et al*, 1993). It is also highly conserved among multiple strains of VV as well as in other orthopoxviruses, including variola virus (Engelstad and Smith, 1993). B5R protein is a type I membrane protein (Engelstad, *et al*, 1992; Isaacs, *et al*, 1992). Its ectodomain is

comprised of four domains with similarity to short consensus repeats (SCRs) and a “stalk” of 51 amino acids located adjacent to the transmembrane region. Recently, epitope-mapping studies have defined two antigenic sites on the B5R protein recognized by their neutralizing activities. They were localized in the SCR1-2 and/or the stalk of the B5R protein (Aldaz-Carroll, *et al*, 2005).

Several researchers have reported that the B5R protein is required for efficient wrapping of IMV, actin tail formation, normal plaque size, and virus virulence (Engelstad and Smith, 1993; Sanderson, 1998). Some laboratories have developed anti-sera against B5R. The developed anti-sera can neutralize EEV infectivity and inhibit the formation of comet-shaped virus plaques (Engelstad, *et al*, 1992; Galmiche, *et al*, 1999; Law and Smith, 2001; Aldaz-Carroll, *et al*, 2005). It has also been reported that the EEV-neutralizing antibodies present in VIG, which is a sterile solution of human plasma containing antibodies to VV donated by vaccinated persons against smallpox virus, are mainly against B5R proteins (Bell, *et al*, 2004). MAbs raised against B5R protein are mainly responsible for the EEV-neutralizing capacity (Bell, *et al*, 2004). It is still unclear why sometimes the developed MAbs or polyclonal anti-sera against B5R protein did not show neutralizing effect but were able to block comet formation (Ichihashi and Oie, 1996; Vanderplasschen, *et al*, 1998b). The possible explanation is that the antibodies were probably not directed against the specific protective epitope(s) in B5R protein, or the protective epitope is not immunogenic during the natural infection or during vaccination using whole virions (Galmiche, 1999). Another explanation is because the EEV and IMV have different mechanisms of cell entry, as mentioned above. Once in the endosome, the fragile outer membrane of EEV is disrupted by the acidic environment,

leading to the release of infectious IMV, which can then enter the cell where IMV-neutralizing antibodies are no longer effective (Bell, *et al*, 2004). Furthermore, knockout virus mutants lacking the B5R gene showed a small plaque phenotype and produced a significantly smaller number of EEV due to a failure to properly wrap the IMV particles, resulting in a greatly attenuated infectivity *in vivo* (Galmiche, 1999). All these evidence indicated that B5R protein is an essential envelope protein which is able to elicit specific neutralizing antibodies against EEV and effectively block the EEV release from the host cell, therefore preventing the virus from further dissemination.

1.4 Vaccinia versus Smallpox infection

The most widely used virus for smallpox vaccination has been the VV. Other species of orthopoxviruses include cowpox (used by Dr. Edward Jenner, 1700s), monkeypox, and camelpox, among others. Eighty percent of variola virus's ORFs possess 90% or greater sequence identical to those of VV (Massung, *et al*, 1994). Individuals vaccinated with VV are able to develop a strong antibody response protective against variola virus (Edghill-Smith, *et al*, 2005). That is one of the important reasons why VV has played a central role in the eradication of smallpox virus, conferring long-term immunity to variola virus infection by cross-reacting antibodies.

1.4.1 General information about smallpox vaccination

Since WHO announced the eradication of smallpox virus in 1980, the world-wide vaccination program has been ceased. There are still concerns for public safety and possible risk of poxvirus outbreaks. The younger population under twenty-five years of

age is susceptible to smallpox infection. Currently available vaccine suitable for human use is characterized by unusual complications (CDC, <http://www.bt.cdc.gov/agent/smallpox/vaccination/facts.asp>; Lane and Goldstein, 2003; Wharton, 2003). Also the recent outbreak of human cases of monkeypox (CDC, 1997; Mukinda, *et al*, 1997; Breman and Henderson, 1998) and the potential use of smallpox as a biological weapon present serious threats to global health. To prepare for such threats, we need to build up the stocks of effective vaccine as well as increase the available supplies of neutralizing antibodies for human prophylactic use.

First generation smallpox vaccines, which were used worldwide in the WHO smallpox eradication program in the second half of the nineteenth century, were prepared from lymph collected from the skin of live animals (most often calves or sheep) infected with VV. Although first generation vaccines are still made in some countries, this method of manufacture is highly undesirable, particularly with respect to controlling smallpox virus outbreak (Monath, *et al*, 2004). The Dryvax vaccine, currently licensed in the United States, is a live and infectious VV prepared from calf lymph, administered by using a multiple puncture technique on the upper arm with a bifurcated needle (Pepose, *et al*, 2003). Although the vaccine does not contain variola virus, the immune response to the VV induces a protective response to variola virus and other related orthopoxviruses as well (Lofquist, 2003). Unfortunately, this vaccine brings unusual side effects and complications by comparison to other vaccines routinely used for other diseases (refer to section **1.4.2**).

Long-term research is underway using recombinant DNA technology to develop a safer vaccine that will provide an effective immune response without the undesirable side effects (Ober, *et al*, 2002). Two companies, Acambis (Cambridge, Mass) and Bavarian Nordic (Copenhagen), are funded for 10 million dollars each by the United States government to develop and test a vaccine based on the modified Ankara strain of VV (MVA), which is a non-replicating virus, in mammalian cells (Washington Post, February 26, 2003). In the mean time, two unlicensed smallpox new vaccines have been developed by Acambis and Baxter Pharmaceuticals. Both are using the NYCBH strain of VV, but one is isolated from human embryonic lung cell culture and the other uses African green monkey (Vero) cells (Cono, *et al*, 2003). At this time, it is not known if these vaccines will be more or less immunogenic than the current Dryvax vaccine. Clinical trials are underway to test these new vaccines. Fortunately, before a new vaccine is licensed by the U.S. Food and Drug Administration (FDA) and becomes available for public vaccination, 1:10 dilution ($10^{7.0}$ PFU/ml) of the existing Dryvax can be used and still generate adequate immune responses and help in extending the use of the available vaccine stockpile (Frey, *et al*, 2002).

1.4.2 Adverse reactions/ Side effects of smallpox vaccination

Adverse reactions to the currently licensed smallpox vaccine, Dryvax, have been well documented and ranged from mild to severe reactions. Mild reactions to vaccine include formation of satellite lesions, fever, muscle aches, regional lymphadenopathy, fatigue, headache, nausea, rashes, and soreness at the vaccination site (CDC, <http://www.bt.cdc.gov/agent/smallpox/vaccination/facts.asp>). Severe complications include accidental

implantation involving extensive lesions, eczema vaccinatum, generalized vaccinia, and progressive vaccinia (Lane and Goldstein, 2003). Individuals with eczema or atopic dermatitis and those undergoing immunosuppressive therapy are especially vulnerable to complications of smallpox vaccination and are usually not recommended for vaccination (Wharton, 2003). New and potentially safer smallpox vaccines are currently under investigations to resolve this problem. However, meanwhile, individuals receiving the current smallpox vaccine, particularly people vulnerable to vaccine complications, will continue to be at risk of severe side effects to the vaccine (Shearer, *et al*, 2005).

One of the most thorough surveys of adverse reactions associated with VV vaccines was conducted in the United States and published by the CDC in 1968: Among each million vaccinations in the United States, there are one death, 1.5 progressive vaccinia, 39 eczema vaccinatum, 12 post-vaccinial encephalitis, and 241 generalized vaccinia (Lane, 1970). Adverse events were approximately ten times more common among those vaccinated for the first time compared to re-vaccinees. Fatality rates were also four times higher for primary vaccinees compared to re-vaccinees (Neff and Lane, 1968).

1.4.3 Treatments of side effects of smallpox vaccination

Passive immunization has been shown to provide protection against diseases caused by several viruses, including hepatitis A, hepatitis B, measles, polio and respiratory syncytial virus (RSV) (Chanock, *et al*, 1993). Condie and O'Reilly (1982) demonstrated that the intravenous injection of hyperimmune cytomegalovirus (CMV) IgG in bone marrow transplant recipients resulted in complete protection against the development of CMV infection. The protection offered to infants by transplacentally transmitted maternal

neutralizing antibodies probably functions similar to the prophylactic use of IgGs (Zinkernagel, 2003). Maternal antibodies may attenuate infection during the initial months of life, thereby creating optimal conditions for the infant to develop specific immunity as a result of natural infection or vaccination. Several years since, Conrad and Lemon (1987) reported that prophylactic intra-muscular injection of serum gamma-globulin containing antibodies against hepatitis B surface antigen and hepatitis A viruses provided significant protection against infection for six months if it was administered before exposure. Currently, a humanized MAb against RSV has been in use to protect at-risk infants (The Impact-RSV study group. 1998).

Similarly with those immunoglobulin products above, VIG is one of the three available treatments for specific complications of smallpox vaccination along with cidofovir, and ophthalmic antivirals. It is mostly against the IMV form of the VV (Smith and Law, 2004). Although VIG has not been tested in controlled clinical trials for efficacy against vaccinia infection yet, it is the first-line therapy because worldwide historical experience with the use of VIG to treat VV related adverse events exists (CDC, February, 2003). The CDC (USA) has contracted a Canadian company, Cangene Corporation, to produce VIG from the serum antibody of VV immunized donors (Winnipeg Free Press, August 13, 2002).

VIG has demonstrated its efficacy in the treatment of eczema vaccinatum, serious generalized vaccinia, and serious manifestations of inadvertent inoculation such as ocular vaccinia (Kempe, *et al*, 1956; Sussman and Grossman, 1965; Sharp and Fletcher, 1973; Goldstein, *et al*, 1975; Cono, *et al*, 2003). VIG is also indicated for progressive vaccinia

and has variable effectiveness in its treatment (Bray, 2003; Hopkins and Lane, 2004). However, since post-vaccinial encephalitis is not due to virus multiplication, VIG is not effective in treating such adverse reaction (Goldstein, *et al*, 1975; Cono, *et al*, 2003).

Current supplies of VIG are limited, and must be used under an Investigational New Drug (IND) protocol (CDC, <http://www.bt.cdc.gov/agent/smallpox/vaccination/mgmt-adv-reactions.asp>). VIG should be reserved for treatment of vaccine complications with serious clinical manifestations. Also, VIG administration has been reported associated with adverse reactions (Cono, *et al*, 2003), for example, an acute allergic reaction to thimerosal, which is one of the components in the VIG solution (CDC, February 2003; Cono, *et al*, 2003). Also, administration with human blood products containing IgA may cause anaphylactic reaction in selective IgA deficient patients (CDC, January 2003; Cono, *et al*, 2003). In a rabbit model of vaccinia keratitis, administration of large or multiple doses (2.5 - 5 times of recommended doses for humans) of VIG was associated with persistent corneal scarring (Fulginiti, *et al*, 1965). No studies have reported the adverse effects of VIG on the fetus or pregnant women yet. Also, whether VIG is present in breast milk is unknown. Therefore, administration of VIG to a pregnant woman is not strongly recommended unless it is clearly needed (Cono, *et al*, 2003). Moreover, the prophylactic use of VIG to treat patients has the potential for the transmission of unknown pathogens or pathogens that we cannot test for at this time. Additionally, VIG contains antibodies of multiple specificities and isotypes. Problems associated with the use of immune globulins for therapeutic applications include lot-to-lot variations and low content of specific antibodies (Slade, 1994).

To reduce the risk of transmitting infectious agents, stringent measures are applied for the selection of blood donors. Restricted standards are used at plasma-collection centers, testing laboratories, and blood fractionation facilities (CDC, February, 2003). However, those safety-screening procedures present a big financial pressure on the government.

Other treatment, such as cidofovir, which is a nucleotide analogue of cytosine made by Gilead Sciences (Foster City, California, USA), has demonstrated antiviral activity against certain orthopoxviruses (Bray, *et al*, 2000; Smee, 2001; Cono, *et al*, 2003). However, its effectiveness on human in the treatment of smallpox vaccination complications is unclear, but its nephrotoxic effect to humans and carcinogenicity to laboratory animals have been reported (Cono, *et al*, 2003). So, cidofovir is not an optimal option unless VIG is not efficacious.

1.5 Monoclonal antibody technologies

It has been about 100 years since Von Behring and Kitasato received the first Nobel Prize for the discovery of passive immunotherapy. And about 30 years since, Kohler and Milstein first reported hybridoma technology. A number of discoveries and technologies have contributed to the applications of antibodies for therapeutic purposes decades after. The use of antibodies as research tools, disease prevention, detection and treatment, has been revolutionized by these recent advances (Gavilondo and Larrick, 2000).

The production of MAbs through cell hybridization is able to break the complex antibody responses into their individual components by using simple cell fusion techniques and standard *in vitro* tissue culture systems. There are two important biological components

in this procedure: B cells from immunized animals and compatible lymphoid-original tumor cell lines named myelomas. Spleen cells from the immunized animals are fused with myeloma cells to create hybridoma cells with properties of both parent cell types. They are able to grow unlimitedly and produce specific single type antibody in tissue culture or when grown as ascites tumors *in vivo*. Applying with appropriate selection and screening methods, it is possible to produce MAbs against any antigen using the MAb production techniques. Since the MAb is a completely homogeneous population, their use in laboratory tests incurs fewer problems with cross-reactivity than conventional polyclonal anti-sera. Applications of MAbs are promising in many areas of medicine and biological science.

Since the discovery of the hybridoma technology, the use of MAbs has become routine in the research and diagnostic laboratory, but antibodies have yet to be used to their maximum potential in medical and public health applications. Two review articles on the therapeutic use of antibodies suggest that systemically administered MAbs may play an important role in treating infections (Casadevall and Scharff, 1995; Casadevall, 1996). Nowadays, MAbs are starting to replace anti-sera, which have been used for years to treat infectious diseases, offering substantial advantages in terms of their potency, specific activity, suitability for a large scale production of homogeneous MAbs and free of contamination with other pathogens (Casadevall, 1996). The production of human MAbs (hMAbs), which is achieved *in vitro* by either hybridoma technology, Epstein-Barr Virus (EBV) B cell immortalization or new recombinant DNA techniques, can provide an unlimited supply of homogeneous product that could entirely overcome most problems associated with VIG (Masuho, 1988). In terms of the specificity, MAb recognizes one

epitope and has markedly higher specific activity than polyclonal antibodies. For example, 0.7 mg of two human MAbs to tetanus toxin has the same activity as 100 to 170 mg of immune globulin (Lang, 1993). The higher specific activity of MAbs may also be translated into greater therapeutic efficacy. It is true that for some infections, polyclonal preparations may be superior to MAbs because polyclonal antibodies contain antibodies to multiple epitopes. However, different therapeutic MAbs can be combined to generate polyvalent preparations composed of predetermined antibodies with multiple specificities and isotypes. The first MAb produced for therapeutic application is the palivizumAb (Synagis), a MAb to RSV which is a pathogen that can cause severe illness in newborn infants (Simoes, 1999). There are relatively few MAbs available against VV, thus there is a need to develop more MAbs against the epitopes on VV that can be used for therapeutic or prophylactic applications (WHO, 2000).

1.6 Objectives of this project

The objective of this project is to produce a number of murine HCLs secreting MAbs able to neutralize VV. The developed MAbs may have therapeutic applications and may be used to develop a rapid diagnostic test for the detection of VV viral infection. To reach this objective, we need to:

- 1). Develop a large panel of murine HCLs producing MAbs against VV;
- 2). Select the HCLs producing neutralizing MAbs against VV;
- 3). Purify and characterize the developed neutralizing MAbs.

CHAPTER 2 MATERIALS AND METHODS

2.1 Virus and cell lines

VV (WR strain) used in our laboratory was kindly provided by Dr. J. Berry (the National Microbiology Laboratory, Winnipeg, Manitoba, Canada). Three different cell lines were used to propagate the virus needed for our study: HeLa cells, CEF primary cells and Vero cells. The HeLa and Vero cell lines are commonly used continuous cell lines. They were maintained in the Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO, Burlington, Ontario, Canada) supplemented with 10% fetal bovine serum (FBS) (HyClone / Fisher Scientific Ltd., Ontario, Canada) in T-75 tissue culture flasks and cultured in a 5% CO₂ incubator at 37°C. Confluent monolayer cells were routinely trypsinized and split 1:10 into new T-75 flasks.

The CEF cell line is a primary cell line derived from chicken embryos. It was prepared following a procedure developed by our laboratory. Briefly, 14 - 15 day old chicken embryonated eggs were bought from the Animal Sciences Department of the University of Manitoba. The chicken embryos were eviscerated aseptically and chicken heads, feet, wings and any feathers were removed. The embryos were minced into small pieces and forced through a 20cc sterile syringe without needle, then incubated in approximately 10 ml of pre-warmed trypsin-EDTA (0.25%) at room temperature for 30 minutes. The supernatants were decanted through 8 layers of sterile gauze into a centrifuge tube and sufficient inactivated FBS was added to constitute 10% of the final volume. The trypsinized tissues were centrifuged at 150 g at 4°C for 10 - 15 minutes. The cell pellets were washed 2 - 3 times and resuspended in plain DMEM medium. The cells were

counted and cell suspensions of 8×10^5 cells per ml were prepared. Each ml of cell suspension was seeded into a T-150 tissue culture flask with 40 ml DMEM containing 10% FBS and incubated in a 5% CO₂ incubator at 37°C.

Both HeLa and CEF cell lines were used for WR VV propagation and purified VV stocks preparation. VV propagated in CEF cells was used to immunize the BALB/c mice and VV propagated in HeLa cells was applied in ELISA and WB assays needed for the screening and characterization of the developed HCLs as indicated bellow. Vero cell line was used for virus titration and PRAs.

2.2 Vaccinia virus manipulation

2.2.1 Purification

VV was purified as indicated by Weisz and Machamer (1994) with some modifications. Briefly, sub-confluent HeLa or CEF cells in T-150 tissue culture flasks were infected with VV diluted in 5 ml DMEM medium at multiplicity of infection (MOI) equal to 0.1 - 1.0 plaque forming unit (PFU) / cell and incubated 1 hour for virus absorption with gentle shaking every 15 minutes. Then 30 ml of DMEM growth medium (DMEM medium containing 2% FBS) was added to each flask. After 3 - 5 days post-infection and when 4+ cytopathic effect (CPE) (about 95% cells became infected and showed CPE) was reached, the virus was harvested. Infected cells were subjected to freezing at -20°C and thawing at room temperature a total of 3 times to break cell membranes and release IMV particles. VV and cell debris were pelleted by centrifugation at 200 x g for 5 minutes at 4°C. Cells pellet was resuspended in 5 ml DMEM medium and homogenized on ice in a

7 ml dounce piston for 40 - 46 strokes using a tight glass pestle. The homogenate was then centrifuged for 5 minutes at 200 x g at 4°C to pellet the cell nuclei and the pellets were washed with DMEM medium twice. All the supernatants were combined and spun down at 650 x g for 10 minutes at 4°C to get rid of any remaining cell debris. The supernatants were then sonicated in a water bath sonicator and overlaid on top of 18 ml of 36% (w/v) sucrose cushion prepared with 1 mM Tris-HCl (pH9.0) in SW 28 centrifuge tubes (Beckman™, Mississauga, Ontario, Canada). The tubes were centrifuged at 32,860 x g in a Beckman™ ultracentrifuge (Model L8-70M) for 80 minutes at 4°C. The purified VV pellet was resuspended in phosphate-buffered saline (PBS) supplemented with 1% penicillin and gentamicin (PENGEN) (Sigma-Aldrich Canada Ltd. Oakville, Ontario, Canada) and 0.5% mycomycin (MYCO, Sigma). Virus aliquots were used for protein concentration assay, virus titration and electron microscope (EM) examination. The rest of the virus suspension was stored at -70°C for later use.

2.2.2 Virus titration by plaque assay

Ten-fold serial dilutions of the purified VV starting from 10^{-5} to 10^{-9} were prepared in DMEM growth media. Each dilution was prepared by using a fresh pipette tip to prevent any crossover between dilutions.

Eighty to ninety percent sub-confluent Vero cells in six-well tissue culture plates were prepared. Tissue culture medium was removed from each well and replaced by 400 µL of each virus dilution. The virus titration was performed in duplicates each time. Vero cell monolayers in two negative control wells were incubated with DMEM growth medium alone. The virus along with the controls was allowed to adsorb to the Vero cells for one

hour at 37°C with gentle mixing every 15 minutes. The inoculae were then removed and replaced with 2 ml of DMEM growth medium. The plates were incubated in the 5% CO₂ incubator at 37°C for 2 - 3 days. Then, supernatant from each well was removed, and plaques were visualized by staining with 1% crystal violet in 2% ethanol. The virus titer was calculated as PFU/ml based on the number of plaques and the dilution factors which gave 10 - 100 plaques/well.

2.2.3 Virus protein concentration assay

The protein concentration of the purified VV was measured using *DC* protein assay kit (Bio-Rad Laboratories Canada Ltd., Mississauga, Ontario, Canada) as indicated in the “microplate assay protocol”. In summary; five serial bovine serum albumin (BSA) dilutions (0.2 mg/ml to 1.36 mg/ml) in PBS were freshly prepared each time for the protein concentration standard curve. Ten µL of BSA standards, virus samples or PBS (as negative control), were added into a clean and dry 96-well micro-titer plate. To each well, 25 µL of reagent A was added, followed by 200 µL of reagent B. Reaction mixtures were mixed by gentle agitation and incubated at room temperature for about 15 minutes for color development. The absorption was measured at 650 nm by using the Dynatech MRX plate reader (Dynatech Laboratories Inc, Chantilly, VA, USA). Standard curve was prepared by plotting BSA concentrations *versus* 650 nm absorptions. The protein concentrations of samples were calculated according to the standard curve and their absorptions at 650 nm.

2.2.4 BEI inactivation of purified vaccinia virus

For safety of the staff of the animal care facility, the Animal Care Committee of the University of Manitoba requested that we inactivate the VV before immunizing BALB/c mice.

The virus was inactivated using a method developed by Bahnemann (1990) with some modifications. Briefly, 10 mM BEI was prepared freshly in each experiment. 0.1 M 2-bromoethylamine (BEA, Sigma) was dissolved in 0.175 N NaOH and incubated at 37°C for 30 - 60 minutes to allow formation of BEI. The formation of BEI was verified by measuring the pH drop from 12.5 to 8.5. For virus inactivation, one volume of freshly prepared 10 mM BEI was added to nine volumes of pre-sonicated purified VV and incubated at 37°C.

Five different incubation periods; 6, 10, 18, 24 and 36 hours were tested to select the optimum incubation time required for complete virus inactivation. After BEI treatment, virus suspensions were dialyzed in 10,000 MWCO Slide-A-Lyzer dialysis cassettes (Pierce Biotechnology Inc. Rockford, IL, USA) against PBS overnight at 4°C, with changing buffer after the first 4 - 8 hours. The dialysis step was added to eliminate any possible toxic effect of the BEI on the injected animals. The toxicity derived from the inactivation reagents and virus infectivity after inactivation-dialysis steps were tested by carrying out plaque assays on Vero cells using neat and 1:10 diluted virus samples. The infectious WR VV before inactivation was used as a positive control. The integrity of the inactivated virus was verified using EM and SDS-PAGE protein gels.

2.2.5 Electron microscope examination of negatively stained vaccinia particles

The purified VV from section **2.2.1** was diluted 1:10 or 1:100 in 1mM Tris buffer containing 2% paraformaldehyde and allowed to stabilize on ice for at least 10 minutes. Forty-five μ L of fixed VV suspension was then centrifuged directly onto formvar coated, carbon-stabilized 400-mesh copper EM grids for 30 minutes at 26-28 psi (Airfuge®, EM-90 rotor, BECKMAN™). The virus samples were then negatively stained with 2.5 mM phosphotungstic acid (pH7.0) and viewed in a Philips 201 EM operated at an acceleration voltage of 60 Kev at magnifications between 20,000X and 82,000X.

2.2.6 Analysis by Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Thirty μ L of purified or inactivated WR VV in PBS from section **2.2.4** was mixed with 10 μ L of 4 X sample loading buffer (1 M Tris, pH6.8, containing 10% sodium dodecylsulfate, 50% glycerol, 0.01% bromophenol blue and 5% β -mercaptoethanol). The viral proteins were denatured by incubating the samples in boiling water for 5 minutes before loading on the gel. The virus samples and molecular weight marker (high-range, Bio-Rad) were then loaded on a 12.5% SDS-PAGE gel (16 cm \times 16 cm \times 0.75 cm). Viral proteins were separated by electrophoresis at 20 mAmps/gel for 3 - 5 hours or 10 mAmps/gel overnight. Separated viral proteins were then stained by Coomassie blue and followed by destaining the background using destain solution (20% methanol and 7.5% acetic acid diluted in distilled water). The SDS-PAGE gel was dried using a gel dryer (Bio-Rad).

2.3 Immunization and hybridoma cells development

2.3.1 Immunization protocol

Four female BALB/c mice (4 - 6 weeks old) were immunized with BEI inactivated VV diluted in PBS. Mice were injected subcutaneously (SC) with 0.1 ml of virus (30 - 40 µg of protein) emulsified with equal volumes of TiterMax[®] Gold research adjuvant (Sigma). Immunization protocol was repeated every 4 weeks until high neutralizing antibody titers were reached in mice sera. Anti-VV antibody titers and the presence of neutralizing antibodies in mice serum were measured using ELISA and PRA respectively before each immunization. Animals with high neutralizing antibody titers were given IP injection using inactivated VV four days before harvesting the spleen.

2.3.2 B cells fusion

Spleen cells were fused with myeloma cells following the standard procedures (Harlow and Lane, 1988; Peters and Baumgarten, 1992) with some modifications. Myeloma SP2/0 cells, provided by Dr. J. Wilkins (the University of Manitoba, Winnipeg, Manitoba, Canada), were recovered from liquid nitrogen one week before use and maintained by 1:2 splitting on a daily basis to make sure that the cells were actively replicating before the fusion. The cells were grown in RPMI 1640 medium (GIBCO) supplemented with 10% (v/v) heat-inactivated high-quality FBS (GIBCO).

The mouse with the highest neutralizing antibody titer against VV was sacrificed by CO₂ asphyxiation. The spleen was removed and blood was collected from the animal heart. A

spleen cell suspension was prepared by gently pressing the spleen through the center of a cell collector (VWR international, Edmonton, Alberta, Canada) using a glass pestle.

Myeloma cells and mouse spleen B cells were washed separately twice with Hanks buffer (GIBCO) and one time with plain RPMI 1640 medium. Cells then were pelleted by centrifugation at 500 x g for 10 minutes. Mouse spleen and myeloma cells, at 2.5×10^7 and 10^8 (1:4 ratio) respectively, were mixed and centrifuged at 400 x g for 5 minutes. Supernatant was completely removed and 1.5 ml of 50% (w/v) polyethylene glycol (PEG) (Sigma) solution was added within 45 seconds while incubating in a 37°C water bath. Then 7 ml plain RPMI 1640 medium was added drop-wise using a syringe fitted with an 18G needle over a 1.5-minute period in different speeds from slow to fast. Then about 42 ml plain RPMI 1640 medium was mixed with cells using a pipette. The cells were spun down at 400 x g for 5 minutes. Cell pellet was resuspended in 190 ml fusion medium (RPMI 1640 medium supplemented with 15% FBS, 10% CPSR-3, 1% 40 uM Aminopterin and 1% HT medium supplement (Sigma) with working concentration of 100 uM Hypoxanthine and 16 uM thymidine). Two hundred μ L of cell suspension was added to each well of fifteen 96-well tissue culture plates and incubated at 37°C in a 5% CO₂ incubator. After 11 - 14 days, supernatants from wells that had visible hybridoma cells were screened for the secretion of antibodies against VV using ELISA, followed by PRA.

2.2.3 Screening for anti-VV antibodies

2.3.3.1 ELISA testing

96-well micro-titer plates (Corning Inc. Life Sciences / Fisher Scientific Ltd., Ontario, Canada) were coated with about 1 μg (or 1.25×10^6 PFU) of purified infectious VV propagated from HeLa in 100 μL PBS per well and incubated overnight at 4°C. The concentrated infectious VV was sonicated for 3 times just before use. Unbound virus was removed by washing once with PBS containing 0.1% v/v Tween-20 (Wash Buffer). The plate was blocked using 300 μL blocking buffer (1% BSA in PBS) per well for two hours at room temperature or 37°C in a humid environment. Aliquots of 90 μL tissue culture supernatant from each well that had hybridoma cells were added to the ELISA plates. 1:500 diluted mice sera in PBS from pre- and post- immunization were included in each ELISA test as negative and positive controls respectively. The plates were then incubated at 37°C for two hours and washed three times using wash buffer. One hundred μL of 1:2000 diluted rabbit anti-mouse IgG (whole molecule) alkaline phosphatase (AP) conjugate (Sigma) in blocking buffer was added to each well. The plates were incubated for 1 hour at 37°C, and then washed 5 times with washing buffer. Two hundred μL of AP substrate (Bio-Rad) was added to each well, and incubated for 30 to 60 minutes at room temperature in dark. The intensity of the developed color was measured as absorption at 405 nm using the Dynatch MRX ELISA plate reader.

In order to test for the possible false positive results caused by reactions between antibodies secreted by HCLs and the blocking buffer, the major 75 positive HCLs from WR VV specific ELISA experiments (indicated above) were tested against the blocking

buffer coated ELISA plates. The rest of ELISA steps were the same as the WR VV ELISA described above. In this BSA specific ELISA, fresh fusion medium was used as negative control.

2.3.3.2 Virus neutralization abilities of the ELISA positive hybridoma cells by plaque reduction assay

The abilities of the secreted antibodies from HCLs to neutralize the WR VV were measured using PRA. To try to keep the cell number and antibody amount consistent, the candidate HCLs were cultured until all the cells died. The culture-to-die supernatants were used in PRA.

A 10^3 PFU/ml of WR VV suspension was prepared in DMEM growth media. Two hundred μ L of the virus suspension was incubated for one hour at 37°C with equal volume of hybridoma cell culture supernatants. DMEM growth media alone and VV suspension in antibody-free DMEM growth media were included as negative and positive controls respectively. Virus titers in the different specimens were measured using plaque assay procedure as indicated in section 2.2.2. The percent of plaque reduction in the virus incubated with hybridoma supernatants compared to the positive control was used as a measure of antibody neutralization.

2.3.4 Hybridoma expansion and sub-cloning

Antibody-secreting hybridoma cells were expanded step-wise from 96-well tissue culture plates (Corning) until they were fully grown in T-25 flasks. Three vials of each HCL were stored in liquid nitrogen before starting the sub-cloning procedures.

HCLs producing neutralizing antibodies were sub-cloned by limiting dilution method by Coller and Coller (1983). Positive colonies were recovered from the liquid nitrogen and resuspended in 10 ml feeding medium (RPMI 1640 medium supplemented with 15% high-quality FBS, 1% 100 X HT medium supplement and 20% conditioned medium). Cells were counted on a hemacytometer. Cell concentration was adjusted to 10^4 - 10^5 cells/ml with feeding medium. Two-fold serial dilutions of the hybridoma cells were distributed in a 96-well tissue culture plate as shown in Figure 2.1. Ten days after plating out the cells, 100 μ L of medium supernatant aliquots, removed from wells with observable single colony, were tested for antibody secretion using ELISA. Sub-cloning was repeated until all the colonies obtained, after limiting dilution, were screened as anti-VV antibody positive.

Conditioned media, which is derived from the naïve mice spleen cells, is one of the important components of the feeding medium. It provides numbers of growth factors and other necessary materials from mice lymphocytes required for the *in vitro* tissue culture of HCLs. The brief procedure to make mice conditional media is as followed: female naive BALB/c mice (4 - 6 weeks old) were sacrificed by CO₂ asphyxiation. The spleens were removed. Spleen cells suspension was prepared by gently pressing the mouse spleens through the center of cell collector using a glass pestle. Mice spleen cells were washed twice in Hanks' buffer and twice with RPMI 1640 medium supplemented with 15% FBS by centrifugation at 500 g for 8 - 10 minutes each wash. The cell pellet from each mouse was resuspended in 100 ml of RPMI 1640 medium supplemented with 15% FBS in a T-75 tissue culture flask and incubated at 37°C in a 5% CO₂ incubator with the flask upright position. After four days, the spleen cells were removed by centrifugation

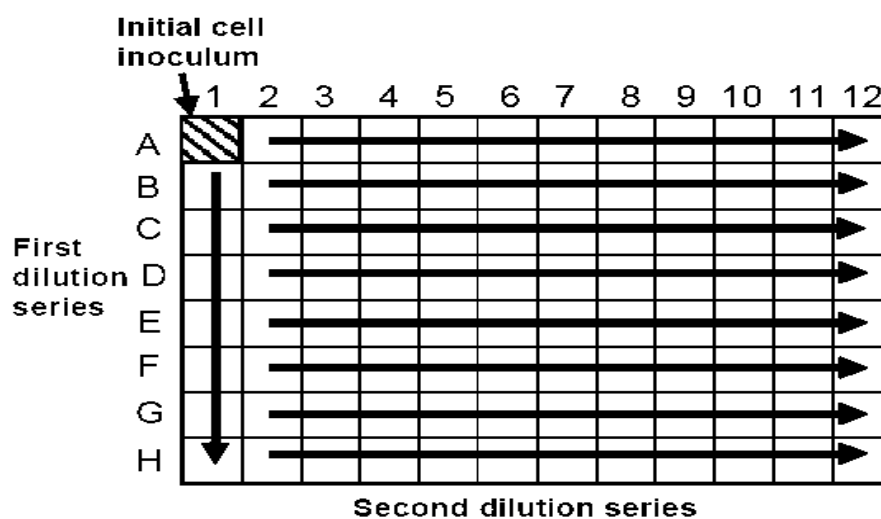


Figure 2.1 Sub-cloning by limited dilution method.

A 96-well tissue culture micro-titer plate is used for sub-cloning process. One hundred μL of initial hybridoma cell suspension, adjusted to the concentration of 10^4 - 10^5 cells/ml, was added to well A1. A two-fold serial dilution was carried out in the first column from well A1 to H1. Then the second 1:2 serial dilution was performed in another direction from column 1 to 12 using a multi-channel pipettor.

and the supernatants were filtered through 0.2 micron sterile filters. Conditioned medium was stored at -70°C for later use.

2.4 Determination of immunoglobulin isotypes

To reach high antibody titers, aliquots of the sub-cloned HCLs producing neutralizing antibodies were cultured in a small amount of feeding medium until all the cells died. Supernatants were clarified by centrifugation and used for antibody isotyping. Antibody isotypes were determined using Mouse Monoclonal Antibody Isotyping Kit (Amersham Biosciences Inc. Baie d'Urfe, Quebec, Canada) according to the kit insert.

In summary; 3 ml of 1:10 diluted supernatant in TBS-T buffer (0.8% NaCl, 0.02 M Tris-base, and 0.3% Tween-20, pH 7.6) was incubated with one isotyping stick for 15 minutes at room temperature with agitation. The typing stick was then washed twice by TBS-T buffer for 5 minutes each time, and incubated with 3 ml of 1:500 diluted peroxidase labeled anti-mouse antibody in TBS-T for 15 minutes at room temperature with agitation. The stick was washed twice with TBS-T, and then 3 ml of freshly prepared substrate solution (one tablet of 4-chloro-1-naphthol dissolved in 10 ml of cold methanol mixed with 50 ml TBS-T and one drop of 30% v/v hydrogen peroxide solution) was added to each stick and incubated for 15 minutes with agitation. The isotyping sticks were washed with distilled water to stop the reaction and the locations of purple color lines developed indicated the antibody isotype and light chain type.

2.5 Monoclonal antibody concentration and purification

The IgG1/ κ MAb purification was achieved with the help of Dr. J. Berry's laboratory. Purification was carried out using 1 ml Amersham HiTrap Protein G column. Tissue culture supernatants with high neutralizing MAb titers were concentrated by ultra-filtration and diluted 1:1 in ImmunoPure (A) IgG binding buffer (Pierce). Protein G column was rinsed using 5 ml of binding buffer and then loaded with 1 ml of concentrated MAb sample. The columns were rinsed with 5 ml of binding buffer to remove unbound proteins. The adhered antibodies were eluted with 10 ml ImmunoPure IgG elution buffer (Pierce) and collected as 10 fractions in tubes containing 100 μ L of binding buffer. The column was then rinsed with another 5 ml elution buffer and 20% ethanol respectively right before storage. Protein concentration was determined in each fraction by measuring light absorption at 280 nm. The appropriate fractions were then retained, pooled and diluted ~5:1 with PBS. Purified MAb sample was then finally concentrated by ultra-filtration using CentriprepTM (Millipore Corp Canada Ltd., Cambridge, Ontario, Canada).

2.6 Characterization of purified monoclonal antibodies

2.6.1 SDS-PAGE analysis

The SDS-PAGE analysis procedure of the purified MAbs was performed as indicated in section 2.2.6. The amounts of purified MAbs loaded on the gel were between 10-20 μ g.

2.6.2 Western blot assay using Enhanced Chemiluminescent (ECL) substrates

SDS-PAGE separated VV proteins (as indicated in section **2.2.6**) were trans-blotted to a 0.2 µm nitrocellulose membrane (Bio-Rad) by the trans-blot semi-dry transfer cell (Bio-Rad) at 15-20 volts for 0.5 - 1 hour. Free sites on the membrane were blocked with 5% powdered skim milk in PBS at 4°C overnight or 1 - 2 hours at room temperature. The membrane was cut into narrow strips used for WB. Purified MAbs were diluted 1:20 – 1:100, based on their concentrations, in PBS and applied to the nitrocellulose strips and incubated for 2 hours at room temperature. Unbound samples were removed by washing the strips 5 times with PBS. The strips were then incubated with 1:5000 diluted secondary antibody, goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Amersham), in PBS for 1 hour and unbound second antibody was removed by washing at least 5 times with PBS. Antigen–antibody interactions were visualized by the treatment with the Western lightning® WB chemiluminescence reagent *plus* (PerkinElmer Canada Inc. Woodbridge, Ontario, Canada), followed by exposure to the KODAK Bio-Max Light Film (VWR).

To verify that the four MAbs developed in this study are specific against VV proteins but not cell debris present as a contaminant from the inoculum, WB was also performed with mock-infected CEF cell lysate following the same procedures as indicated above.

2.6.3 MAbs dose-dependent effect on WR VV neutralization

To investigate the quantitative relationship between WR VV neutralization and developed MAbs, PRA was performed, as indicated in section “**2.3.3.2**”, using different

amounts of purified MAbs in the range of 0.01 - 10 µg prepared in DMEM growth medium.

2.6.4 Synergistic effects of mixing two different MAbs on WR VV

Synergistic effects among different MAbs on WR VV neutralization were tested by mixing two different MAbs at the amounts giving about 20% or 40% plaque reduction in PRA for each. The same amounts of each single MAb were used as controls. The neutralizing abilities of the MAb mixtures, along with controls, were measured using PRA with the same procedures as indicated in section “2.3.3.2”. MAb mixtures that gave higher plaque reductions by comparison to their controls were considered to have synergistic effects.

2.6.5 Plaque reduction assays against other vaccinia strains

Three VV strains, L-variant (VR-2035), NYCBH (VR-1536) and IHD-W (VR-1441), were obtained from ATCC. They were propagated in HeLa cells and titrated using plaque assay on Vero cells. The abilities of four purified MAbs to neutralize the three VV strains were tested by PRAs following the same procedures as indicated in section “2.3.3.2” using four different MAb doses: 0.01, 0.1, 1 and 10 µg.

CHAPTER 3 RESULTS

3.1 Vaccinia virus purification

Highly purified WR VV was needed in our study to immunize BALB/c mice as well as to develop serological assays to screen the developed MAbs. Based on the virus propagation and purification protocols used in our study, we were able to prepare stocks of purified WR VV required for our study. A virus concentration of 10^9 - 10^{10} PFU/ml was achieved according to plaque assay on Vero cells. The purified WR VV was viewed by EM examination. Comparing the WR VV before and after the sucrose cushion step, EM results (Fig 3.1) demonstrated that the purified virus samples from CEF or HeLa cells contained much less cellular debris.

3.2 Vaccinia virus inactivation

The effects of five different incubation time periods of WR VV with 10 mM BEI on virus inactivation were tested. Results from plaque assay and EM micrographs indicated that 24 hours was the optimal incubation time (Table 3-1). We were able to reach 100% virus inactivation and good virus integrity after incubating the virus for 24 hours at 37°C and then dialyzing overnight against PBS at 4°C.

Complete virus inactivation with no residual effect of BEI was verified by plaque assay on Vero cells. No CPE or any cell toxicity was detected after infection for 2 - 3 days at 37°C (Fig 3.2 B and C). However, the positive control: Vero cells infected with up to 10^{-8} dilution of the purified VV before inactivation, showed clear CPE 2 - 3 days post-infection (Fig 3.2 D-F). The effect of BEI on the structure of the inactivated VV

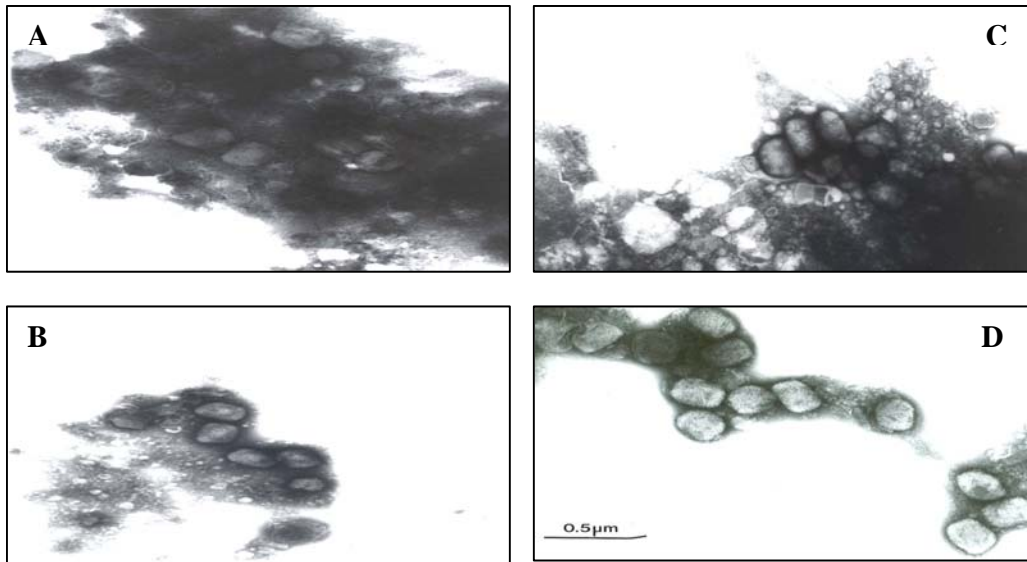


Figure 3.1 Electron micrographs of vaccinia virus before and after purification.

VV propagated in HeLa and CEF cells. Infected cells harvested at 4+ CPE were frozen/thawed 3 times. Cell debris was removed by centrifugation at 200 x g. The virus suspension was then layered on a 36% w/v (in 1 mM Tris) sucrose cushion and centrifuged at 32,860 x g for 80 minutes. Virus pellet was resuspended in PBS. Purified virus at 1:10 and 1:100 dilutions was fixed with 2% paraformaldehyde. EM grids were prepared by air-fuge, negatively stained and viewed under EM. (A) and (B): VV propagated in CEF cells before and after sucrose cushion purification; (C) and (D): VV particles grown in HeLa cells before and after sucrose cushion purification. EM micrographs indicated that the purified VV from either HeLa or CEF cells had much less contaminant by comparison to the virus before the sucrose cushion step.

Table 3-1 Optimization of WR VV inactivation conditions by 10 mM BEI using five different incubation time periods and PBS dialysis method

Incubation (BEI+VV)	CPE on Vero (3 days pi)	Toxicity on Vero		Effect on virus integrity
		before dialysis	after dialysis	
6hr	yes	yes	no	no
10hr	yes	yes	no	no
18hr	sometimes	yes	no	no
24hr	no	yes	no	no
36hr	no	yes	no	yes*

** Too long time incubation with BEI will result in the breakage of virus particles and increasing debris in virus sample under EM examination.*

To optimize the BEI inactivation condition on WR VV, five different incubation periods at 37°C: 6, 10, 18, 24 and 36 hours, were tested. Dialysis of inactivated virus samples against PBS was carried out at 4°C overnight. The toxicity of samples before and after the dialysis step, and virus infectivity after inactivation were tested by monitoring cell death and plaque formation on Vero cells using plaque assays. The integrity of the inactivated virus was verified using EM and SDS-PAGE. Each incubation time was tested three times.

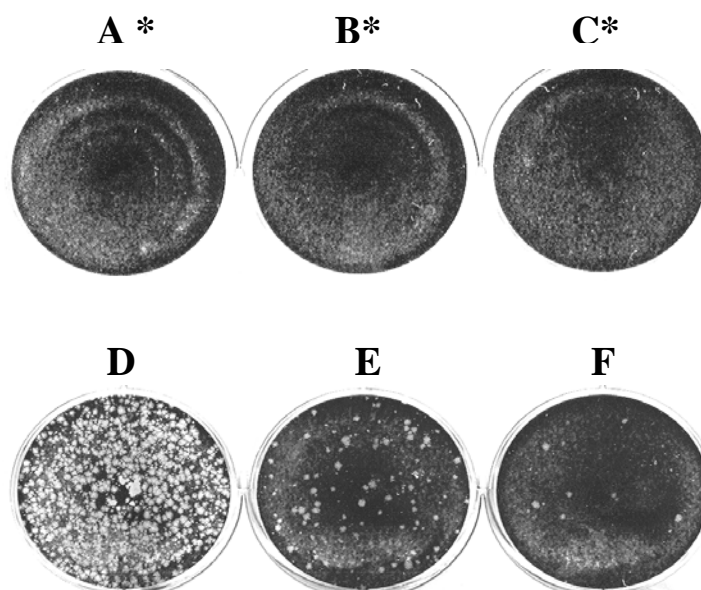


Figure 3.2 Plaque assay of vaccinia virus infectivity after BEI inactivation

Plaque assay was performed using inactivated VV. (A) Negative control: DMEM growth medium alone. (B) and (C) WR VV after inactivation: neat and 1:10 diluted respectively. (D) to (F) Positive controls: serially diluted (10^{-6} to 10^{-8} from left to right) infectious VV before inactivation.

** The light dots in (A)-(C) were not actual plaques caused by viral infection, but the spaces among Vero monolayer cells, which were not stained by crystal violet.*

was determined using EM examination. There was no visible morphological difference between VV particles before and after inactivation (Fig 3.3). The same conclusion was also obtained by SDS-PAGE analysis, which indicated that there was no difference in the electrophoresis patterns between VV before and after inactivation and dialysis (Fig 3.4).

3.3 Hybridoma development

3.3.1 ELISA results

Spleen cells from four BALB/c mice were fused with murine myeloma SP2/0 cells using PEG method. There were more than 3,000 HCLs detected when tissue culture plates were examined using inverted microscope. Of them, 261 HCLs were secreting antibodies against VV proteins using in-house WR VV specific ELISA (Fig 3.5). The antibodies were considered recognizing VV proteins if the ELISA OD values were greater than 2X OD value of the negative control. Although many of the tested HCLs gave OD values as low as the negative control, there were some with OD values close to or even higher than the positive serum control.

To confirm that the antibodies produced were specific for VV proteins but not to BSA which was used to block the ELISA plates, BSA-specific ELISA was performed. Seventy-five VV- specific-ELISA highly positive HCLs (from above) were tested against BSA. All their OD values were lower than 0.4 (Fig 3.6), which was less than two times the negative control, indicating that there was no false positive in VV specific ELISA indicated in Fig 3.5.

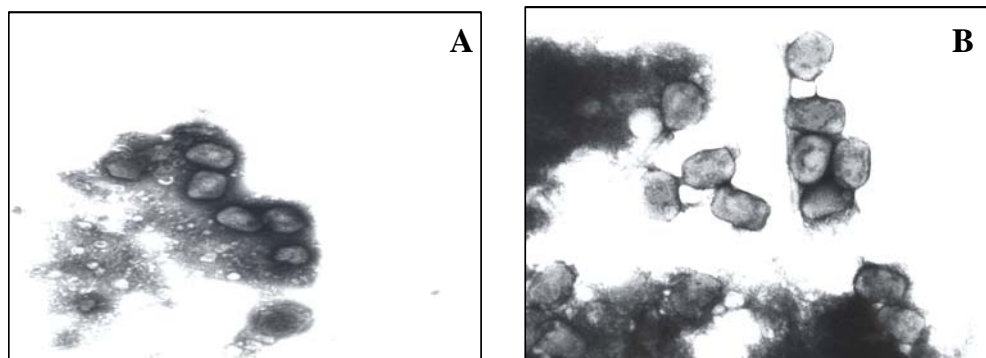


Figure 3.3 Electron micrographs of infectious (A) and inactivated (B) WR VV particles derived from CEF cells.

VV before (A) and after (B) BEI inactivation were 1:100 and 1:10 diluted in 2% paraformaldehyde respectively. Virus samples were centrifuged onto EM grids and negatively stained by phosphotungstic acid. There was no significant difference on the morphology of VV between EM micrographs A and B, indicating that at least the outside structures of viral particles were still intact and inactivation did not affect VV envelope.

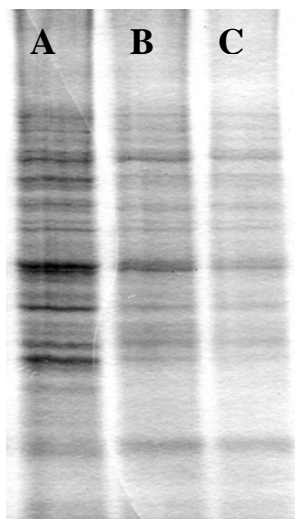


Figure 3.4 SDS-PAGE gel analysis of WR VV proteins before and after inactivation.

A 12.5% SDS-PAGE gel was prepared for analysis of the viral protein before and after BEI inactivation. Three WR VV samples: before, after BEI inactivation and after PBS dialysis overnight, were loaded using 20, 15 and 10 μg respectively. Lane A: sucrose purified infectious WR VV particles derived from CEF culture; Lane B: WR VV virus from step (A) was incubated with 10 mM BEI for 24 hours; Lane C: BEI inactivated VV after PBS dialysis process. The similar viral protein patterns in SDS-PAGE gel between the infectious and inactivated VV, although quantity differences of some proteins bands exist probably due to the dialysis process, indicated that BEI inactivation did not have any significant effect on the viral protein. This result is consistent with EM results indicated in Fig 3.3.

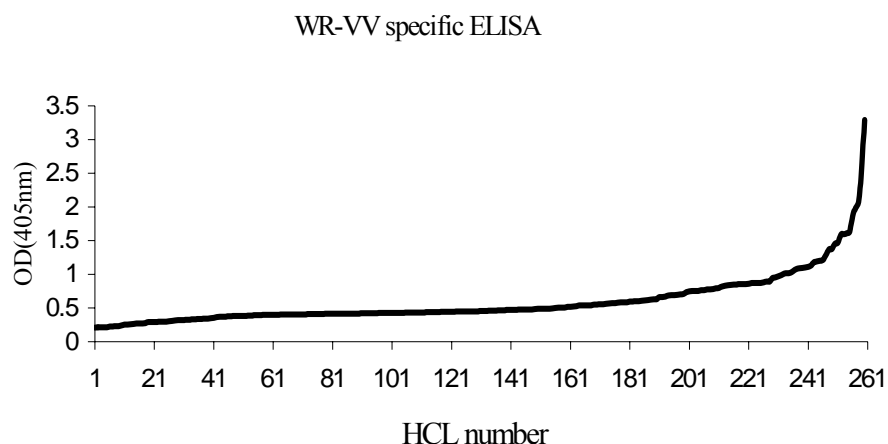


Figure 3.5 Display of the WR-VV specific ELISA results from four different fusions.

The HCLs were screened using ELISA plates coated with HeLa-derived infectious WR VV at 1.25×10^6 PFU or $1 \mu\text{g}$ per well. Mice sera (1:500 diluted in PBS) from naïve and immunized were included as negative and positive controls respectively. Out of over 3,000 hybridoma cells, 261 HCLs were ELISA positive. Each HCL was considered positive if its OD value $\geq 2X$ of the negative control (0.13). The OD values of positive HCLs at 405 nm were distributed in a wide range from ~ 0.3 to 3.5. The graph indicates all the positive HCLs values tested by ELISA.

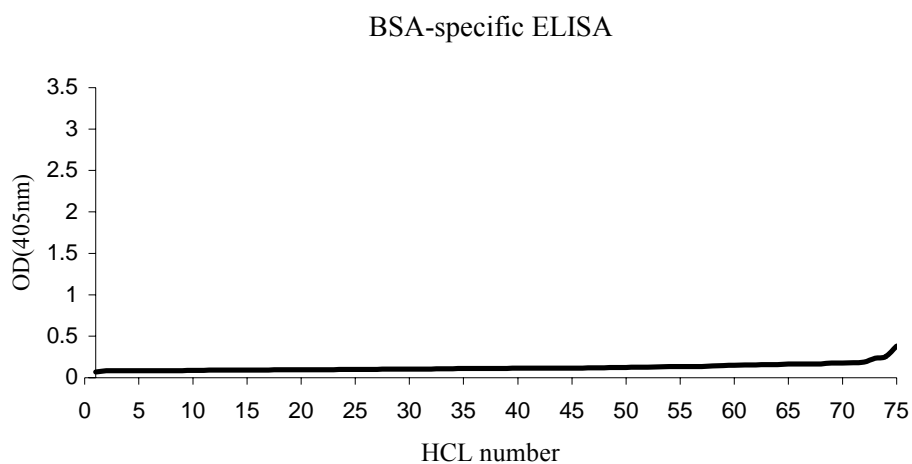


Figure 3.6 Display of the BSA-specific ELISA results from four fusions.

To exclude the possibility of false positive results caused by reaction to the blocking buffer, the tissue culture supernatants of the 75 most highly positive HCLs from Fig 3.5 were tested with blocking buffer coated ELISA plates. There was no BSA positive OD value obtained (the OD value of negative control is 0.22), reflecting that all the positive HCLs were specifically against WR VV, and no false positive results presented in Figure 3.5.

3.3.2 Plaque reduction assays

The HCLs producing antibodies against VV were tested for their abilities to neutralize the WR VV infection using PRA. Sixty-five of the 261 ELISA positive HCLs were secreting neutralizing antibodies. The antibodies were considered neutralizing if they reduced the virus infection by at least 10% in three consecutive PRA repeats. Using 200 μ L of tissue culture supernatants from some positive HCLs in the 96-well tissue culture plates, before any antibody purification or concentration, were able to neutralize up to 63% of the 200 PFU WR VV used in the assays (Fig 3.7).

3.3.3 Sub-cloning and isotyping

Twelve HCLs secreting the highest titers of neutralizing antibodies (Fig 3.8) were sub-cloned using limiting dilution method. Seventy-nine sub-clones were developed from 10 HCLs. The other two HCLs died during the expansion and before the sub-cloning step. Sixty-six of 79 sub-clones displayed neutralizing abilities similar to or higher than their parental HCLs. Sixty (90%) of 66 MAbs were IgG1/ κ isotype. Isotypes of the other 6 MAbs include IgG2b/ κ , IgG3/ κ and IgM/ κ (Fig 3.9).

3.4 Characterization of the four purified MAbs

3.4.1 SDS-PAGE analysis

The four most neutralizing MAbs (IgG1/ κ isotype) were purified from tissue culture supernatants of candidate clones. The four MAb clones, 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6, 2-F6-11-G6, came from two different immunized BALB/c mice. SDS-PAGE analysis

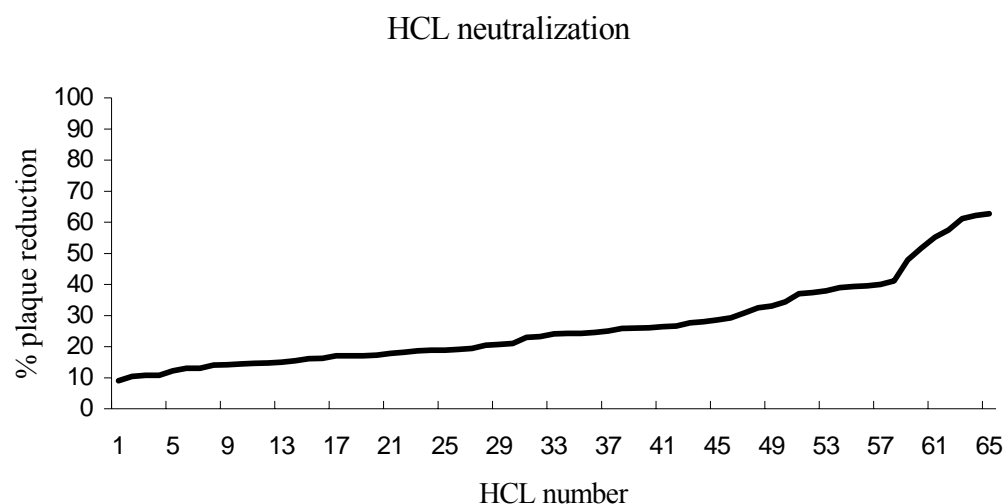


Figure 3.7 Plaque reduction assay results of the HCL tissue culture supernatants from four fusions.

The VV-specific ELISA positive HCLs were further screened by PRA for their neutralizing abilities. Briefly, equal volumes of each HCL tissue culture supernatant and 200 PFU of WR VV in DMEM growth medium were mixed and incubated at 37°C for one hour. The virus-supernatant mixture was then used to infect Vero cells monolayers. The antibody neutralization ability was calculated by plaque reduction of the mixture in relation to the positive control which was VV only. Sixty-five out of the 261 ELISA positive HCLs displayed neutralizing abilities against WR VV by PRA in the range of 10% - 63%. Each PRA result represents the mean of three experiments.

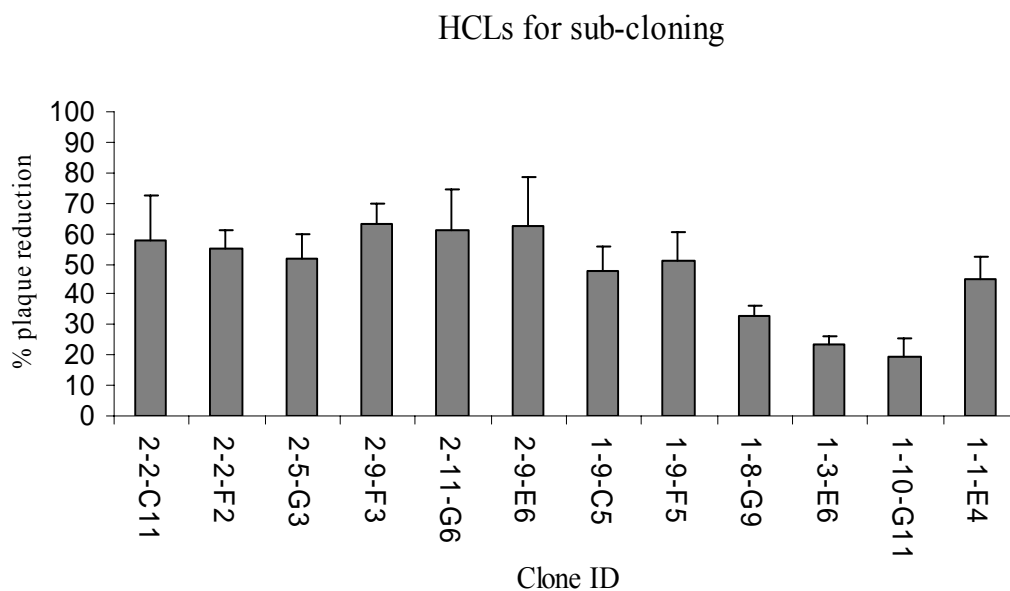


Figure 3.8 Twelve highly neutralizing hybridoma cell lines selected for sub-cloning.

The most neutralizing HCLs from four different fusions are listed. The VV neutralization of different HCLs ranged from 20% - 63% when using 200 PFU of WR VV in each test. Error bars are indicated for three replicates. The samples used by PRA here were tissue culture supernatants. The HCLs were further sub-cloned by limited dilution method to make MAbs.

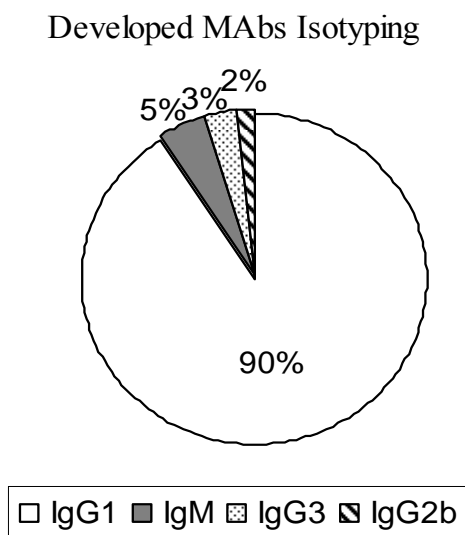


Figure 3.9 Isotyping of 66 sub-clones derived from 10 highly neutralizing HCLs.

Sixty-six neutralizing sub-clones were obtained by sub-cloning ten highly neutralizing HCLs. The isotypes of the developed MAbs secreted from these clones included IgG1, IgG2b, IgG3 and IgM. Among them, 90% (60 MAbs) were IgG1/ κ isotype.

indicated (Fig 3.10) that each of the four MAbs has a 55 kDa protein, which represents the IgG1/ κ isotype heavy chain. Moreover, the four MAbs have slightly variable light chains, the low molecular weight bands in the gel, in the range of 27-33 kDa. This variation may reflect that the four MAbs are different in their variable domains of the light chains and they may recognize different epitopes.

3.4.2 Western blot assay results

VV propagated in HeLa cells was purified on a 36% sucrose cushion and trans-blotted to a nitrocellulose membrane after separating the denatured virus proteins in a 12.5% SDS-PAGE. Four different MAbs were tested with such membranes by WB to determine their target viral proteins on WR VV. Each of them recognized the same three VV proteins with molecular weights of 75 kDa, 45 kDa and 35 kDa (Fig 3.11 A1-4). Interestingly, MAb clone 2-B7-9-E6 reacted with one extra 8 kDa protein (Figure 3.11 A3). According to our PRA results as described in section **3.4.3.1**, MAb 2-B7-9-E6 has the highest VV neutralization ability among all the tested MAbs, reflecting that this extra small viral protein may play an important role in WR VV neutralization.

To confirm that the developed MAbs were not reacting with CEF cell debris present in the inoculum, WB was also performed with mock-infected CEF cell lysate. The results indicated that the 4 MAbs did not recognize any CEF proteins (Fig 3.11 B1-4). On the other hand, the positive serum control, sera from the immunized mice, reacted with some CEF proteins (Figure 3.11), which was expected since the mice were immunized with virus preparation containing some cell debris.

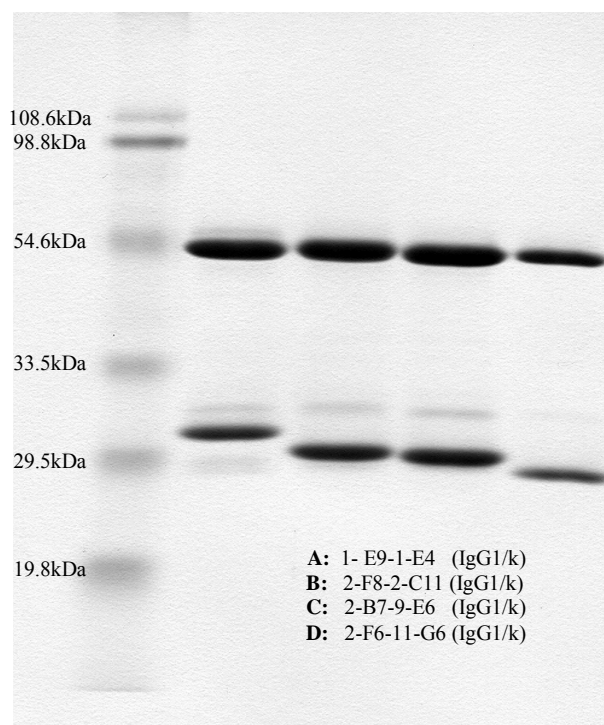


Figure 3.10 SDS-PAGE analysis of four purified MAbs (IgG1/k).

Ten μ g of each MAb, mixed with 4X sample buffer, denatured by incubation in boiling water for 5 minutes and separated in a 12.5% SDS-PAGE. The protein bands were stained with Coomassie blue. The four MAbs have similar high molecular weight bands around 55 kDa, which represents the heavy chains of IgG1 isotype. There were some variations on the mobilities of their low molecular weight bands, in a range of 28 - 31 kDa, which may indicate some difference in the variable regions of the light chains.

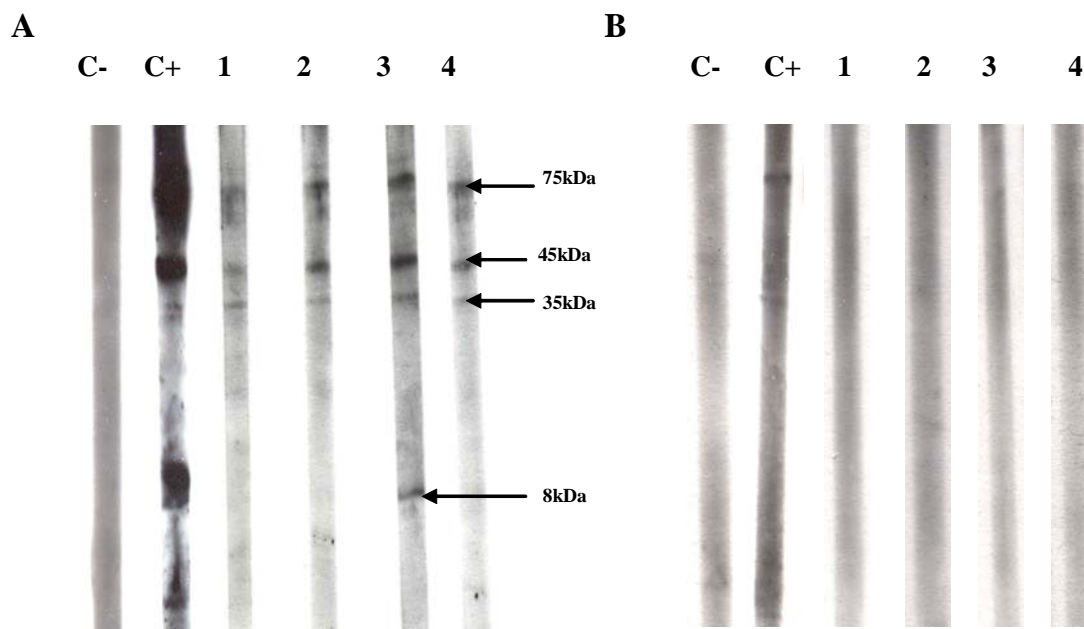


Figure 3.11 Western blot assay results of the four purified MAbs.

(A) WB using WR VV trans-blotted to nitrocellulose membrane strips. (B) WB on the mock-infected CEF cell lysate trans-blotted nitrocellulose strips. In both (A) and (B), the negative and positive controls were 1:100 diluted naïve and immunized mice sera respectively. The four purified MAbs, **1:** 1-E9-1-E4; **2:** 2-F8-2-C11; **3:** 2-B7-9-E6; **4:** 2-F6-11-G6, were 1:20 - 1:100 diluted in PBS.

3.4.3 Plaque reduction assays on WR VV

3.4.3.1 MAb dose effect on neutralization of WR VV

Two hundred PFUs of purified WR VV were incubated with five doses (0.001 to 10 µg) of each purified MAb. The virus neutralization at each MAb dose was measured using PRA. Our results indicated that neutralization abilities of the developed MAbs were correlated with the MAb amount used in the tests and their maximum neutralization levels varied from 70% to 95% with the fact that MAb clone 1-E9-1-E4 gave the least neutralization and MAb clone 2-B7-9-E6 gave the highest neutralization (Fig 3.12 A-D). Fifty percent of neutralization (neutralization titer) was estimated at 0.01, 0.02, 0.04 and 0.34 µg of MAbs 2-B7-9-E9, 2-F8-2-C11, 2-F6-11-G6 and 1-E9-1-E4 respectively based on their neutralization curves (Fig 3.12 E).

3.4.3.2 Synergistic effect of mixing two different MAbs on neutralization

We studied the synergistic effect on the neutralization of the VV WR strain by mixing 2 different MAbs. Increases on VV neutralization were detected by mixing the MAbs 1-E9-1-E4 and 2-B7-9-E6 at the doses able to neutralize about 20% and 40% VV in PRA (Fig 3.13). The three mixtures, $A_{20}B_{20}$, $A_{40}B_{20}$ and $A_{20}B_{40}$ neutralized the virus infection by 71%, 68% and 78% respectively, while the plaque reduction % of controls: $A_{20} + B_{20}$, $A_{40} + B_{20}$ and $A_{20} + B_{40}$ were 43%, 56% and 70% respectively. The virus neutralization increased 8% to 28% by mixing these two MAbs. However, there was no increase in neutralization detected in mixture $A_{40}B_{40}$ or by mixing other sets of MAbs.

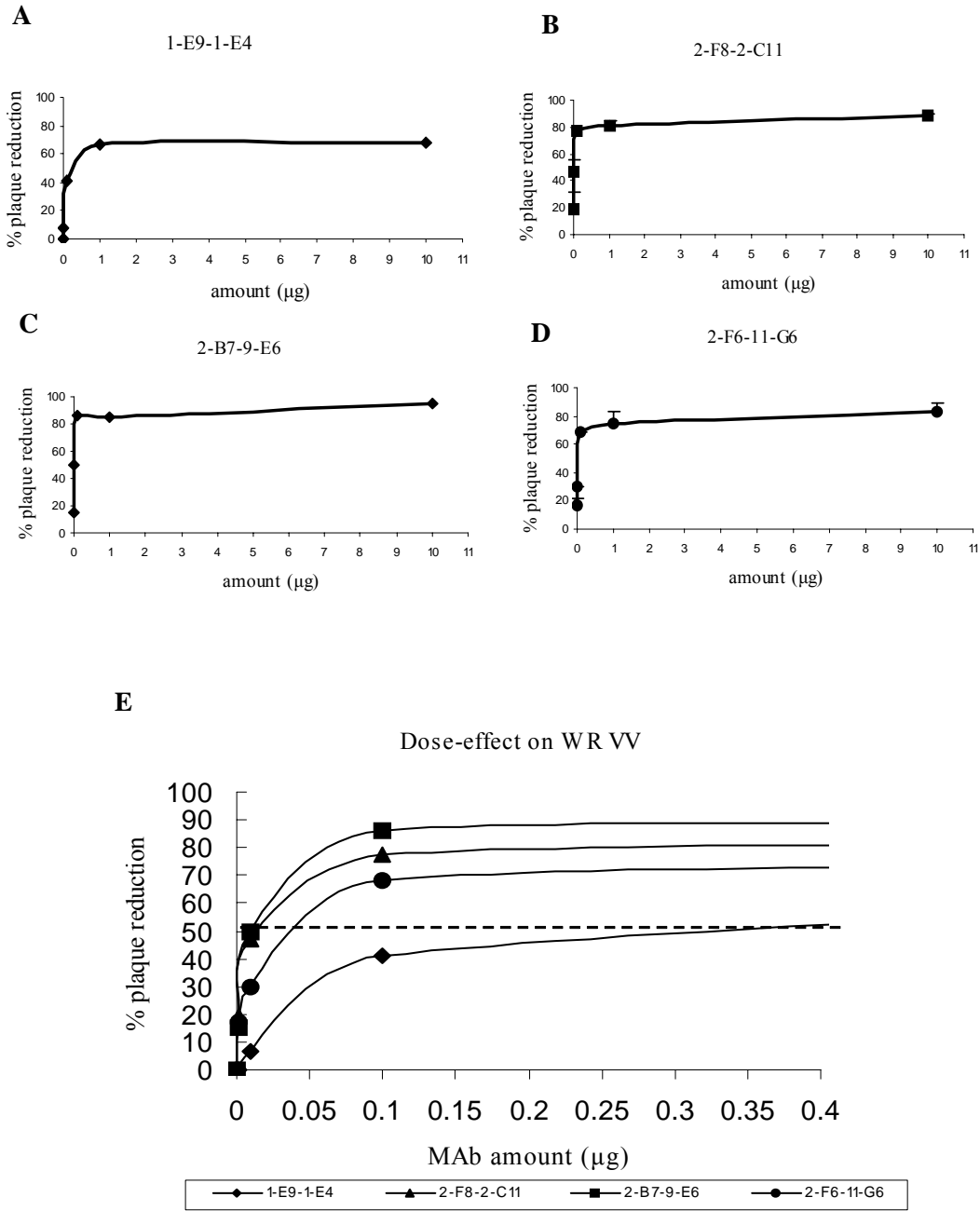


Figure 3.12 Dose dependent neutralization of 4 purified MAbs against WR VV.

To determine the MAbs dose effect on WR VV neutralization, five different amounts of each MAb: 0.001, 0.01, 0.1, 1.0 and 10 μg , in DMEM growth medium were used in PRA. (A) - (D) are dose-dependent neutralization curves of four purified MAbs: (A) 1-E9-1-E4; (B) 2-F8-2-C11; (C) 2-B7-9-E6; and (D) 2-F6-11-G6. Each point represents the average of three replicates. Error bars are indicated. (E): Displaying of MAbs neutralization curves at lower antibody concentrations to estimate their neutralization titers. Based on curves (A) – (D), the 50% neutralization titers of MAbs, 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6 and 2-F6-11-G6, were estimated at 0.34, 0.02, 0.01 and 0.04 μg respectively.

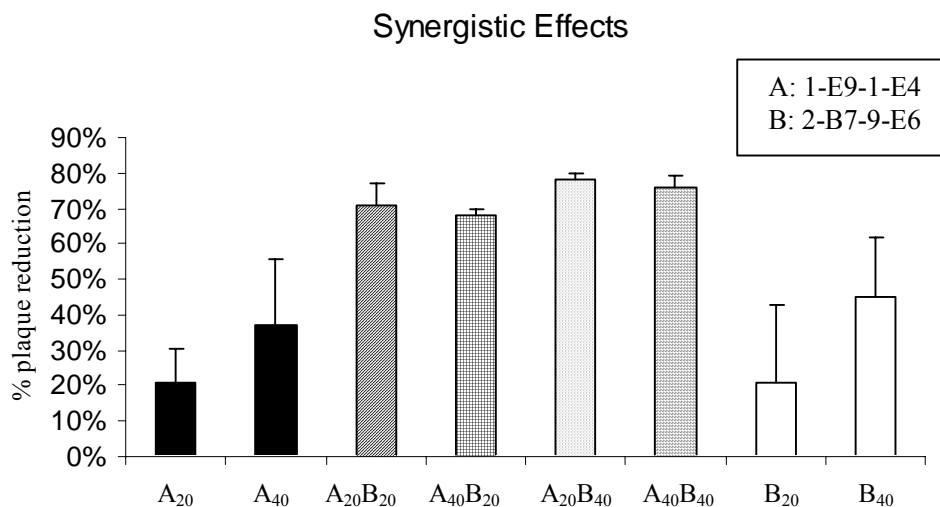


Figure 3.13 Synergistic effect results by mixing MAbs 1-E9-1-E4 and 2-B7-9-E6.

PRA results of mixing two MAbs, 1-E9-1-E4 and 2-B7-9-E6, at amounts capable of neutralizing about 20% (A₂₀ and B₂₀) and 40% (A₄₀ and B₄₀) VV neutralization were shown as % plaque reduction. Each result was the average of three different assays. The two single MAbs at the amounts that gave about 20% or 40% plaque reduction were used as controls represented by the solid-color histograms. Synergistic effects were measured by comparing the % plaque reductions between MAbs mixtures and the controls. Synergistic effects were observed in mixtures A₂₀B₂₀ (71%), A₄₀B₂₀ (68%) and A₂₀B₄₀ (78%). Neutralization abilities of those mixtures increased by 8% to 28% compared to the controls: A₂₀ + B₂₀ = 43%, A₄₀ + B₂₀ = 56% and A₂₀ + B₄₀ = 70%.

3.4.4 Neutralization against other strains of vaccinia virus

There are many different strains of VV, and some have been used to develop smallpox vaccine. Thus, it is interesting to test the neutralization abilities of the developed MAbs on other VV strains.

Three VV strains NYCBH strain, IHD-W strain, and L-variant strain were tested with the four MAbs developed in our laboratory. The MAbs displayed variable levels of neutralizing abilities on three such VV strains. The variations may relate to the differences in the envelope proteins among different VV strains.

3.4.4.1 Plaque reduction assay on L-variant vaccinia strain

Neutralization of the four developed MAbs against L-variant VV showed a very similar dose-dependent pattern as WR VV (Table 3-2). Clone 1-E9-1-E4 gave the least neutralization and clone 2-B7-9-E6 gave the highest neutralization among the four MAbs. The maximum neutralization abilities of the different MAbs varied from 60% to 80% (Fig 3.14 A). Neutralization titers of the four MAbs were estimated at 0.11, 0.057, 0.042 and 0.095 μ g for clones 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6 and 2-F6-11-G6 respectively (Fig 3.14 B) based on their neutralization curves. The similarity in neutralization between L-variant and WR VV indicates that L-variant and WR VV strains are closely related.

3.4.4.2 Neutralization on NYCBH vaccinia strain

The neutralizations of the 4 MAbs against NYCBH VV, as shown in Table 3-3, were in the range <10% - 30%, which was remarkably lower than either WR or L-variant VV at the same dose. Neutralization was only observed using 1 µg and 10 µg of MAbs. The low but insignificantly different PRA results, between the two doses, reflected that the neutralization against NYCBH VV may be in a distinct manner from WR and L-variant VV strains by the developed MAbs.

3.4.4.3 Plaque reduction assay on IHD-W vaccinia strain

There was almost no neutralization against IHD-W VV from PRA results: only 0-11.25% plaque reductions were detected when the high MAb doses (10 µg) were used (Table 3-3). But interestingly, 2-B7-9-E6, which was always the most neutralizing MAb in our study, did not show any neutralization. However, 2-F8-2-C11 and 2-F6-11-G6 were able to neutralize IHD-W VV weakly (10% - 20%).

Table 3-2 Plaque reduction assay results of 4 purified MAbs at four doses against L-variant vaccinia strain.

MAbs	10µg	<i>S.D.^a</i>	1µg	<i>S.D.^a</i>	0.1µg	<i>S.D.^a</i>	0.01µg	<i>S.D.^a</i>
1-E9-1-E4	61.5%	1.34	63.2%	4.19	49.7%	3.74	6.7%	4.56 ^b
2-F8-2-C11	78.3%	7.21	70.4%	6.35	62.4%	7.56	20.0%	3.81 ^b
2-B7-9-E6	70.7%	2.68	75.2%	6.58	71.1%	2.89	26.7%	1.90
2-F8-11-G6	68.2%	5.44	60.2%	5.03	50.8%	5.37	6.0%	3.48 ^b

a. *S.D.* stands for “Standard Deviation”. The values indicated in the table are means of three replicates of PRA results.

b. Since 0.001 µg is a very low MAb dose, minor changes in PRAs may lead to significant deviations in the results. So, the *S.D.* of PRA results were expected high at 0.001 µg.

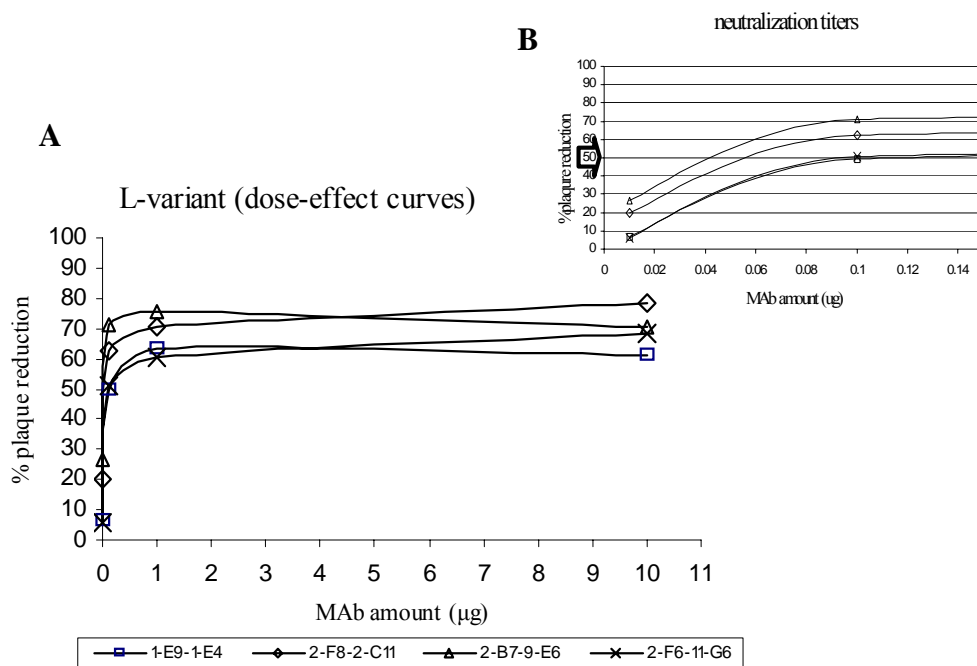


Figure 3.14 Dose-dependent neutralization curves of four MAbs against L-variant vaccinia virus.

Based on the PRA results at four different MAb doses against L-variant VV from Table 3-2, the dose-dependent neutralization curves of four MAbs were obtained, as indicated in (A). 50% neutralization titer of each MAb was estimated from curves at lower doses, shown in (B). Based on curves, neutralization titers of four MAbs against L-variant VV are estimated at: 0.11, 0.057, 0.042 and 0.095 μg for MAbs 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6 and 2-F6-11-G6 respectively.

Table 3-3 Neutralization of the 4 purified MAbs against NYCBH and IHD-W vaccinia strains

	NYCBH (1 µg)	<i>Standard Deviation</i>	NYCBH (10 µg)	<i>Standard Deviation</i>	IHD-W (10 µg)	<i>Standard Deviation</i>
1-E9-1-E4	9.4%	0.67	8.1%	0.45	0	0
2-F8-2-C11	23.2%	2.43	28.0%	2.24	6.7%	0.08
2-B7-9-E6	25.7%	0.42	26.0%	0	0	0
2-F6-11-G6	21.3%	2.30	21.0%	2.24	11.3%	1.47

- a.* NYCBH and IHD-W VV strains were propagated in HeLa cells and titrated on Vero cells. The PRA results of four purified MAbs at 1 µg and 10 µg per 200 PFU of NYCBH VV are indicated. Only 10µg dose of two MAbs was able to neutralize IHD-W VV in PRA.
- b.* Each PRA was repeated three times. The values shown in the table are averages.

CHAPTER 4 DISCUSSION

VV infection is known to efficiently trigger both humoral and cellular immunities against smallpox infection. The production of anti-VV antibodies is one of the key components of long-term immune memory against smallpox re-infection. That is also one of the important reasons why VV has been used as a live vaccine against smallpox infection for several decades. However, severe side effects occurred more often than other vaccinations and the vaccination is not recommended for immunocompromised individuals and pregnant women. Passive administration of VIG prior to or during an infection has been shown to reduce the severity of the smallpox disease and to be effective in the treatment of vaccine complications. Currently, large amounts of VIG are under preparation to be used in case of re-emergence of smallpox infection. However, considering the nature of VIG as a human blood product, there are still some concerns using VIG for treatment. So, there is a need for much safer and more effective alternative to VIG. MAbs are a promising choice. They do not have the potential for transmitting unknown pathogens and can be more specific and much easier to produce a homogeneous product on a large scale. Considering IMV VV particles are a major target of VIG and more stable than EEV, we focused on developing MAbs against IMV VV.

The objectives of our study were as follows: To develop neutralizing MAbs against IMV VV in immunized mice by using classic PEG-mediated hybridoma development techniques; To characterize the developed MAbs for their isotypes, their target viral proteins on VV, their abilities to neutralize WR and other strains of VV and the possible synergistic effects of mixing different MAbs on VV neutralization.

4.1 Antigen preparation

4.1.1 WR vaccinia virus purification

High titers of pure VV were needed to vaccinate the animals and coat the ELISA plates required for screening the developed antibodies. We decided to immunize BALB/c mice with purified VV to increase the chance of producing antibodies against VV proteins and reduce the efforts on screening the developed HCLs. In this study, we have established a protocol to purify the intracellular WR VV from different cell lines. Electron micrographs of purified VV propagated in different cell lines demonstrated that WR VV propagated in HeLa or CEF cells had the least cell debris by comparison to the virus derived from Vero cells or chicken embryo chorioallantoic membranes. This observation may be due to the different abilities of the virus to bind to different cell membranes.

As mentioned in CHAPTER 2, we used two different cells lines, CEF and HeLa, to propagate the WR VV for murine immunization and the subsequent antibody screening and characterization assays, respectively. The use of VV from two different cell lines may help to minimize the background cross-reaction with the contaminants in the virus preparation used for mice immunization and ease the selection of antibodies against VV.

4.1.2 Inactivation of WR vaccinia virus

Some investigators reported that they immunized BALB/c mice using different live viruses to achieve good immune reactions during the development of MAbs (Llames, *et al.*, 2000; Singh, *et al.* 2004). It is possible that we may generate a better immune response and higher antibody titers if the BALB/c mice had been immunized with live

VV rather than inactivated virus. However, as indicated by the University of Manitoba Animal Care Committee for safety considerations, we were to use only inactivated virus to immunize animals that were housed in the animal care facility of the University of Manitoba.

Generally, BEI and beta-propiolactone (BPL) are two chemicals commonly used to inactivate viruses for the purpose of developing vaccines suitable for human or animal use (Bahnemann, 1975; el-Karamany, 1987; Buonavoglia, *et al*, 1988; Perrin and Morgeaux, 1995). Based on published data, the majority of the developed neutralizing antibodies are recognizing the epitopes present on the surface of the virus particles. Compared to BPL, which we tried previously on WR VV, BEI is more efficient in virus inactivation, less toxic to cells and less expensive (King, 1991). Importantly, it has been reported that BPL may alter the epitope structures of the surface proteins, whereas BEI does not (Blackburn and Besselaar, 1991; King, 1991; Mondal, *et al*, 2005). Additionally, BEI has been used to effectively inactivate several human viruses, including foot-and-mouth disease virus, rabies virus, sheep pox and VV for vaccine developing purposes (Larghi and Nebel, 1980; Awad, *et al*, 2003; Aarthi, *et al*, 2004). It inactivates virus by directly targeting the nucleic acid without any damage to the viral envelope (Bahnemann, 1975; Larghi and Nebel, 1980). So the use of BEI to inactivate WR VV exhibited several advantages over BPL in this study.

In our study, the integrity of VV after BEI inactivation has been demonstrated by EM and confirmed by SDS-PAGE. Although both EM and SDS-PAGE have their limitations, they were useful to check if there was a major change on the viral morphology or protein

structures as the result of virus inactivation protocol. In the later ELISA screening step, we obtained reasonable number of HCLs producing antibodies reacting with IMV form of infectious VV, which also partially confirmed that the difference on mice immunization between the infectious and inactivated VV particles was insignificant. Furthermore, comparing the use of specific MAbs and immunofluorescence techniques, indicated by Blackburn and Besselaar (1991), with the EM method, used in our study, to determine the effect of BEI inactivation on the immunobiological properties of the VV particles, we preferred the latter due to its simple, fast manipulation, lower expense and the ability to demonstrate the integrity of the virus particles.

EM and plaque assay had demonstrated that incubating the virus with 10 mM BEI for 24 hours was sufficient to inactivate the virus. Otherwise, longer incubation could lead to damage on virus particles. However, the optimum incubation time may vary if different forms of WR VV particles, such as EEV which are more fragile than IMV, or other VV strains were used.

As indicated by Bahnemann (1990), the residual BEI was hydrolyzed with sodium thiosulfate after virus inactivation. However, in our study, we prefer PBS overnight dialysis to remove any BEI residues and prevent any toxic effect that sodium thiosulfate hydrolyzation might bring to the immunized animals or the cells used for plaque assays.

4.2 Development of hybridoma cell lines

Four female BALB/c mice were immunized using the same amount of antigen and the same protocol. The animal with the highest neutralizing antibody titer in its serum was

sacrificed first and its spleen cells were fused with murine myeloma cells. There were differences in the number of HCLs producing antibodies against VV from the four immunized mice as well as the number of HCLs producing neutralizing antibodies. The differences may be due to the biological differences between animals or the random fusion of the spleen cells to the myeloma cells. They also may be because of the differences in the animals' age at the time of harvesting the spleen cells. Older animals gave lower number of hybridoma cells and lower number clones producing neutralizing MAbs. Although we have tried to do the cell fusion under the same conditions in terms of pH, temperature, timing, buffering condition and using the same PEG lot number, *etc.*, minor changes in some of these parameters may still have a major effect on the number of HCLs produced (De StGroth and Scheidegger, 1980).

4.3 Characterization of the four developed MAbs

4.3.1 Isotyping

The isotype distribution of developed MAbs can reflect the level of antibody response by the time mouse spleen was harvested. Based on immunological knowledge, a secondary antibody response should coincide with antibody switching from IgM to IgG isotypes, such as IgG1 and IgG2b in mice. As expected, the majority (about 90%) of the MAbs developed in our study were IgG1 single isotype, indicating a good response induced by immunization. Additionally, the single isotype of each MAb in the result also confirmed the monoclonalities of the developed MAbs.

4.3.2 Analysis of MAbs by Western blot assay

Since the majority of VV used for mice immunization were IMV particles, viral proteins involved in IMV neutralization were crucial in this study. Since the whole genome of VV has been sequenced, all possible proteins are potentially known. In previous studies, as indicated in the “INTRODUCTION” section, several investigators had detected some IMV proteins, such as A27L, A17L, H3L and L1R proteins, important in antibody neutralization of VV infection. In our study, WB results had demonstrated that the four developed MAbs, 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6 and 2-F6-11-G6, targeted the same three IMV proteins, 75 kDa, 45 kDa and 35 kDa. One extra 8 kDa protein was recognized by the highest neutralizing MAb 2-B7-9-E6. According to the protein molecular sizes in SDS-PAGE gel, the 35 kDa and 8 kDa proteins are equivalent to H3L and A13L proteins respectively. There is no literature reporting vaccinia neutralization is related to the 75 kDa and 45 kDa vaccinia proteins. The two proteins can be the precursors of either 35 kDa or 8 kDa proteins, or can be the viral proteins which share closely related epitopes recognized by the developed MAbs in our study. Since it is interesting that the two vaccinia viral proteins, 35 kDa and 8 kDa, detected in our study have the same SDS-PAGE sizes with the well-known H3L and A13L vaccinia proteins, respectively, we will focus on discussing and comparing them next.

H3L protein is a VV protein dominantly expressed on the surface of IMV particles and participates in the attachment of VV to target cells (Da Fonseca, *et al.* 2000a, 2000b). Although a variety of immunogenic VV antigens eliciting neutralizing antibody responses have been reported in the literature, the vast majority of that work was done in

animal models. Interestingly, VV H3L protein has been found as a dominant antigen in the human antibody response and most likely to be a key contributor to the protection against poxvirus infection and disease (Davies, *et al.* 2005). Meanwhile, Davies (2005) reported that H3L VV protein is one of the 14 specific antigens on the VV proteome microarray recognized by VIG. Interestingly, our study has demonstrated that MAbs, which reacted with a 35 kDa VV viral protein, as the same size as H3L protein, also played an important role in neutralization against IMV VV. Most importantly, we have achieved a higher level of VV neutralization using an anti-35kDa MAb that also has the specificity against an 8 kDa viral protein comparing with other MAbs developed against H3L protein (Rodriguez, *et al.*, 1985) in the similar amounts. This result indicated that whole VV-neutralization activity by MAbs may need the presence of several neutralizing antibody specificities, which is in agreement with our following analysis on neutralization in section 4.3.3 and consistent with the published data by Rodriguez (1985) as well.

A13L protein is another abundant protein on IMV membrane (Salmons, *et al.*, 1997) and it has been implicated as non-related to neutralization in previous studies (Pulford, 2004). Interestingly, in our study, an 8 kDa viral protein, the same size as A13L protein, was proved contributing to the neutralization against VV. It is very possible that the two proteins are totally different viral products with different functions. However, it may also because different VV strains were used in the two studies; Pulford (2004) used IHD strain, whereas we used the WR strain. This possibility was partially confirmed by our PRA results mentioned in section 3.4.4; the MAb clone 2-B7-9-E6, which targets the 8 kDa protein of WR VV strain, dose not neutralize the IHD-W VV strain, which is very

close to the IHD strain used by Pulford. This may indicate that A13L protein is not conserved among different VV strains. Additionally, another possibility is that MAbs developed in our and Pulford's laboratories may recognize different epitopes on the same protein. Although two different MAbs may target the same viral protein, it is very possible that they recognize distinct epitopes with significantly different roles in virus neutralization. Last, the synergistic effect of A13L or 8 kDa protein on neutralization function is possible. It may involve or enhance VV neutralization only when react together with proteins recognized by MAb clone 2-B7-9-E6. In another words, the MAb against A13L or 8 kDa vaccinia protein may be able to neutralize VV infection if it recognizes epitopes present on other VV proteins.

4.3.3 Neutralization against WR vaccinia virus

The four MAbs obtained from two separate fusions are highly neutralizing WR VV in a dose dependent manner. Our data also indicated that their neutralization abilities inclined to plateau when over a certain amount. The percentage of virus neutralization varied using same amount of different MAbs indicating that there are differences in their neutralization properties. Interestingly, the use of 10 µg of MAb 2-B7-9-E6 gave >95% neutralization of 200 PFU VV, which is considered a comparable level of neutralization with other MAbs developed against VV (Rodriguez, *et al*, 1985) or even against other human pathogens, such as HIV (Binley, *et al*, 2004).

Neutralization is actually a very complicated process. It has been defined as “the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency. As such this is an unusual activity of

antibody paralleled only by the inhibition of toxins and enzymes” (Dimmock, 1995). The mechanisms involved in neutralization have been debated over years. Based on Klasse and Sattentau (2002), antibody neutralization against VV could happen in one of the following steps during VV replication cycle: the first step may occur during VV attachment to the susceptible cells. Infection can be completely stopped if the antibodies blocked this step. Secondly, the formation of VV-antibody complex aggregates can be another mechanism. The virus aggregations may reduce the virus PFU titer and thus attach to fewer host cells. However, the attachment of aggregates may be stronger and deliver a higher dose of virus to a smaller number of cells than monodisperse virions would. This is consistent with our result mentioned in section 4.1.1 that infection with higher MOI virus produced fewer virus particles than the cells infected with lower MOI. A third mechanism could be that antibody prevents the interactions of the virus with its receptors and co-receptors after the attachment. In this stage of viral life cycle, antibody interference can happen during the VV fusion with the cell surface or in endosomal vesicles. The fourth possibility is the uncoating step, in which, the neutralizing antibodies may inhibit the first metabolic events catalysed by viral enzymes, such as the transcription of VV genome. In most cases, blunting viral infection is the most protective effect. However, Burton (2002) indicated neutralizing antibodies also might act directly against infected cells *in vitro* by binding to the same viral proteins expressed on the cells as on free virus. He also mentioned that non-neutralizing antibodies might contribute to protection in certain instances.

Based on the above theories, the plateau phenomenon occurred in antibody neutralization and the differences between each developed MAb may indicate that VV neutralization is

a multiple-step process. It may have to be accomplished by inhibiting different steps in the virus attachment and internalization. Controlling the different steps to reach complete virus neutralization may require mixing different MAbs to work together. Single MAb may not be able to completely neutralize the virus infection. Also, since the complexity of VV life cycle, the non-neutralizing antibodies might contribute to the whole neutralization process as mentioned by Burton (2002), which could be an important part of the future investigations on MAb neutralization against VV in this study.

Since neutralization seemed to be a multiple-component procedure, we hypothesized that there may be some synergistic effects by mixing different MAbs. Synergistic effects were observed in 3 mixtures of two MAbs. The two MAbs were from two different mice, which makes it very possible that they may recognize two totally different epitopes and can have synergistic effect on each other in VV neutralization. However, the synergistic effects did not display equally when different amounts of MAbs were used. The higher amount of MAbs we combined, the less synergistic effects they had. The possible reason for this phenomenon is that the neutralization had reached its plateau when higher dose of MAbs were used and could not increase as fast as the lower dose, which is consistent with the dose-dependent neutralization results. Meanwhile, it is still interesting to continue investigating more neutralizing MAbs, or study the non-neutralizing MAbs, which may have important roles in protection from VV infection and may contribute to the integrity of the whole neutralization system.

4.3.3 Cross reactivities against other vaccinia strains

In addition to WR VV strain, there are >100 other VV strains, wild and recombinant, available in the world nowadays (information from ATCC). Researchers have used several strains, from less pathogenic NYCBH strain to less infectious strains (such as MVA), to vaccinate people at risk of exposure to smallpox virus infection. Also, there are a number of laboratories using different VV strains, where the accidental exposure is very possible. So, it is interesting and important to detect the cross protections among those strains.

The main goal for smallpox vaccination is to induce a strong and efficient neutralizing antibody response against VV whole particles or virus proteins involved in the early steps of VV infection. Neutralizing antibodies induced by VV vaccine are genus-specific and cross protective among other Orthopoxviruses, such as monkeypox, cowpox, and variola viruses (Jezek and Fenner, 1988; CDC, 1997; Moyer, *et al*, 2000). In our study, we tested the cross-neutralization abilities of the developed MAbs on three other available VV strains: NYCBH, L-variant and IHD-W. Variable neutralization abilities were observed against them. The neutralization abilities of the four MAbs against L-variant, which is a large variant isolated from WR strain (information from ATCC), were similar to WR VV neutralization. However, the neutralizations on the other two strains: NYCBH and IHD-W, were not as significant as the L-variant. They can only be neutralized by high doses of MAbs. The variation in the abilities of the developed MAbs to neutralize different VV strains is most likely due to the slight variations in protein structures among the different strains. The developed MAbs may be more specific to the WR VV strain and its closely

related isolates than being able to neutralize the wide range of VV strains. However, interestingly, the neutralizing manner on IHD-W VV by the developed MAb was different from WR, L-variant and NYCBH strains, the higher neutralizing MAb did not neutralize at all, but the other did, although very weakly. This may be due to the different neutralization mechanisms involved against different strains. We were unable to test the developed MAb against more VV strains since new USA regulations prevent ATCC from shipping VV strains to the laboratories that do not have research project funded by the US Department of Health.

4.4 Future directions

According to what we have finished so far in this project, there are still many interesting issues to investigate in the future:

First, the SDS-PAGE and WB results have indicated that A13L and H3L might be the target proteins recognized by all the four developed MAb and MAb clone 2-B7-9-E6 respectively. Further confirmation of the target proteins should be performed using purified proteins.

Second, as mentioned above, antibody neutralization is a complex process. We have developed several promising neutralizing MAb against WR VV: some of them have synergistic effects and some of them are able to neutralize more than one VV strains. There is a need for further study of the mechanism of MAb neutralization by investigating more of the developed HCLs or highly positive MAb against VV as obtained by ELISA. The non-neutralizing MAb may play important roles in VV

neutralization when they are used with other neutralizing MAbs. Also, testing the neutralization abilities of developed MAbs on more VV strains or even other poxviruses will widen the applications of the developed MAbs.

Additionally, to be able to use the developed MAbs for human clinical applications, it is an important step to humanize the developed murine MAbs, which is our long-term goal to do. The humanized MAbs may be used on the treatment of smallpox vaccination severe side effects and complications instead of VIG, or to develop rapid diagnostic methods for the detection of VV and smallpox infection.

CHAPTER 5 CONCLUSIONS

The objectives of our study were to produce MAbs capable of neutralizing WR VV and characterize them. We have established a simple but efficient WR VV propagation and purification procedure from HeLa or CEF cell lines with high viral titers and purity. Incubation of purified virus for 24 hours with 10 mM BEI was demonstrated to be optimal for WR VV inactivation and meanwhile keeping virus particles intact. In this study, we generated four highly neutralizing MAbs, 1-E9-1-E4, 2-F8-2-C11, 2-B7-9-E6 and 2-F6-11-G6. Their neutralizing abilities were dose dependent and mixing MAbs clones 1-E9-1-E4 and 2-B7-9-E6 displayed synergistic effects. WB results indicated that the four MAbs targeted three same viral proteins, 75 kDa, 45 kDa and 35 kDa, but may recognize different epitopes. We also found that the most neutralizing MAb 2-B7-9-E6 also targeted one extra 8 kDa VV protein. Interestingly, the developed MAbs were able to neutralize other VV strains to different degrees, which may widen the applications of the developed MAbs on either clinical or research aspects in further investigations.

The developed MAbs may be used as a research tool to study VV pathogenesis, to develop chimeric human antibodies for clinical applications, such as treatment of smallpox vaccination severe side effects and complications instead of VIG, or to develop rapid diagnostic methods for the detection of VV and smallpox infection.

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