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DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL
ANTIBODIES DIRECTED AGAINST YABA-LIKE
DISEASE VIRUS

by

Scott Haller

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
requirements for the
Degree of Master of Science
Department of Biological Sciences

Western Michigan University
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August 2001

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2001

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Scott Haller

DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST YABA-LIKE DISEASE VIRUS

Scott Haller, M.S.

Western Michigan University, 2001

The hybridoma technique has been used for the production of monoclonal antibodies (mAb) directed against yaba-like disease virus (YLDV). Spleen cells, obtained from female BALB/c mice immunized with YLDV, were fused with P3x63.Ag8.653 myeloma cells and hybrids were selected. Hybridoma clones were screened for production of anti-YLDV antibodies by Indirect Immunofluorescence (IF) microscopy. Positive hybridoma clones were recloned via limiting dilution and characterized. A single clone (H12) has been selected that specifically reacted with YLDV infected cells and not uninfected cells. Immunodiffusion technique determined H12 monoclonal antibody as an IgG1 isotype immunoglobulin. H12 antibodies recognized a late YLDV protein, and cross-reacted with tanapox virus (TPV) infected cells providing additional evidence to classify YLDV and TPV as different strains of the same virus. The approximate molecular weight of the antigen has yet to be confirmed. The H12 mAb should prove invaluable in the elucidation of the virus-host interactions with YLDV and TPV potential diagnosis of these infections in humans and monkeys.

TABLE OF CONTENTS

ACKNOWLEDGMENTS.....	ii
LIST OF TABLES.....	vi
LIST OF FIGURES.....	vii
INTRODUCTION.....	1
Poxviruses.....	1
Tanapox Virus and Yaba-Like Disease Virus.....	3
Classification.....	3
Epidemiology of TPV and YLDV.....	4
Morphology of TPV and YLDV Virions.....	5
Biological Similarities of TPV and YLDV.....	6
TPV and YLDV Genomes.....	7
Monoclonal Antibodies.....	7
History of Antibody Discovery.....	7
Brief History of Monoclonal Antibody Technology.....	8
Myeloma Cells: Non-Secreting Immortal Fusion Partner.....	9
Techniques for Cell Fusion and Hybridoma Selection.....	10
Applications of Monoclonal Antibodies.....	12
Conclusions.....	16

Table of Contents- continued

MATERIALS AND METHODS.....	17
Virus, Cells and Reagents for Virus Cultivation.....	17
Virus Concentration.....	17
Immunization.....	19
Fusion.....	19
Myeloma Cells and Reagents.....	19
Myeloma Cell Quantification.....	20
“Feeder Cells”.....	21
Fusion Protocol.....	22
Hybridoma Screening Assay.....	24
Collection of Hybridoma Supernatant.....	24
Development of Coverslips.....	25
Indirect Immunofluorescence (IF) Assay.....	25
Maintenance of Positive Clones.....	27
Cloning by Limiting Dilution.....	27
Cryopreservation of Hybridoma Cells.....	28
Test of Cryopreserved Hybridoma Viability.....	28
Hybridoma Characterization.....	29
Hybridoma Supernatant Concentration.....	29
Indirect Immunofluorescence (IF) Assay and Digital Imaging.	30
Hybridoma Isotype Determination- Immunodiffusion Assay..	30

Table of Contents- continued

Immunoprecipitation of Viral Antigen by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis.....	31
Preparation on Radiolabeled Viral Antigen.....	31
SDS-PAGE Resolution of Viral Antigen.....	33
Hybridoma Cross Reactivity Assay.....	35
Determination of Viral Antigen as Either an Early or Late Protein.....	35
RESULTS.....	37
Development of Monoclonal Antibodies Directed Against Yaba-Like Disease Virus.....	37
Hybridoma Clone H12 Produces IgG1 Isotype Antibody.....	39
Indirect Immunofluorescence Microscopy with H12 Hybridoma Supernatant.....	40
H12 Antibody is Directed Against a Late YLDV Polypeptide.....	44
H12 Antibody Demonstrates Cross Reactivity with TPV through Indirect Immunofluorescence Microscopy.....	47
Immunoprecipitation of Viral Antigen with H12 Supernatant Demonstrates Two Polypeptide Chains Precipitated.....	49
DISCUSSION.....	51
APPENDICES	
A. IACUC Approval Letter.....	54
B. Radiation Safety.....	56
BIBLIOGRAPHY.....	58

LIST OF TABLES

1.	Summary of Number of Wells Screened per Fusion with Immunofluorescence Results.....	38
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LIST OF FIGURES

1.	Ouchterlony Double Diffusion Analysis Demonstrating the Isotype of the Antibody Secreted by Hybridoma Clone H12.....	41
2.	Indirect Immunofluorescence Microscopy Demonstrating the Selective Binding of H12 to YLDV Infected Owl Monkey Kidney Cells.....	43
3.	Indirect Immunofluorescence Microscopy Demonstrating H12 Binding Directed Against a Late YLDV Polypeptide.....	46
4.	Indirect Immunofluorescence Microscopy Demonstrating H12 Cross Reactivity with TPV.....	48
5.	Autoradiogram of Immunoprecipitates with H12 Demonstrating Precipitation of Two Polypeptide Chains.....	50

INTRODUCTION

Poxviruses

Poxviruses are the largest and most complex of all known animal viruses. The family *Poxviridae* can be classified into two subfamilies: the *Chordopoxvirinae* (infecting vertebrates), and the *Entomopoxvirinae* (infecting insects). There are eleven genera of the *Poxviridae* family. Viruses that are included in the same genus are related both genetically and immunologically and share a common ultrastructure and host range (reviewed in Moss, 1996).

Poxviruses have contributed significantly in the fields of both biomedicine and molecular biology. Cowpox virus has been used to create a vaccine against smallpox virus and vaccinia virus is used today as a vector for the insertion of foreign genes in vaccine development through recombinant techniques (Mackett *et al.*, 1982).

The entire replication cycle of poxviruses occurs in the cytoplasm of their host cell, making them unique among DNA viruses. All species of poxviruses, except members of the *Yatapoxvirus* genus, produce pocks on the chorioallantoic membrane of embryonated chicken eggs (Fenner, 1996).

Poxviruses are large, complex particles, oval or "brick-shaped" in structure, and measuring 300-400 nm x 170-260 nm. Deharven and Yohn (1966) showed that thin sections under electron microscopy reveal an outer surface composed of lipid and

protein surrounding a biconcave or dumbbell-shaped core. Analysis of vaccinia virus demonstrates that the dry weight of the virion is 90 % protein, 5 % lipid, and 3.2 % DNA (Smadel and Hoagland, 1942, Zartouw, 1964). The double-stranded, linear DNA of poxviruses is found within the core unit. Interestingly, there are two forms of viral particles observed during replication. The intracellular mature virions (IMV), which contain a single inner membrane, and the extracellular enveloped virions (EEV), which contain a second extracellular or outer membrane envelope (Payne, 1978)

Poxviruses are taken up by the host cell through an active process termed pinocytosis (Joklik, 1968) and go through two cycles of uncoating. In the first uncoating process, occurring immediately upon entry into the cytoplasm of the host cell, the viral particle is converted to viral cores (Joklik 1964, Sarov and Joklik, 1972). It is within these viral cores that some early transcription occurs (Kates and McAuslan, 1972, Daul and Kates, 1970a, Daul and Kates 1970b) coding for proteins that are necessary for the second stage of uncoating and viral DNA replication (Dubbs and Kit, 1964, Smith *et al.*, 1989, Slabaugh *et al.*, 1988, and Broyle, 1993). This results in the release of naked viral DNA and initiation of viral DNA replication (Sarov and Joklik, 1972). The viral DNA is replicated in organized areas in the cytoplasm termed viral factories (Fenner *et al.*, 1989). As infection proceeds, these viral factories become larger. Viral assembly also occurs within the cytoplasm of the host cell with two forms of infectious particles seen, those being the IMV and EEV (Payne, 1978). EEV migrate to the cell surface along microfilaments (Hiller *et al.*,

1981), fuse its outer layer with the host cell plasma membrane, and are released by budding (Payne, 1978).

The cytopathic effect (CPE) that is observed from poxvirus infection is dependent not only on the strain of the infecting virion, but also the type of host cell that is infected. The general morphologic cytopathogenesis involves the rounding of infected cells followed by granulation of the cytoplasm due to the developing viral factories. Poxvirus infection also causes the host cell to rapidly alter its shape and cytoskeletal organization (Traktman, 1991).

Tanapox Virus and Yaba-Like Disease Virus

Classification

Tanapox virus (TPV), yaba-like disease virus (YLDV) and yaba monkey tumor virus (YMTV) are the three members comprising the *Yatapoxvirus* genus. *Yatapoxvirus* genus viruses infect all primates, but cause disease primarily in non-human primates. TPV, YLDV and YMTV are related to one another based on size, shape and ultrastructure. TPV is serologically related to YLDV, but restriction maps of the viral genomes of TPV and YLDV reveal a difference in fragment patterns with the restriction endonuclease *Pst*I (Downie and Espana, 1973, Kight *et al.*, 1989).

Epidemiology of TPV and YLDV

TPV was first isolated from a population of natives living within the Tana River Valley in Kenya, Africa in 1962 (Downie *et al.* 1971). This epidemic was associated with a period of extensive flooding in Kenya when the human population together with their domestic and wild animals was crowded on small islands among the floodwaters. In Zaire, cases are reported throughout the year, with the majority of the cases being reported between the months of November and March (Jezek *et al.*, 1985) and in the township of Lisala, among persons living within 300 meters of the Zaire River.

YLDV first gave rise to epizootics in three primate centers in the United States in 1965-66, the National Center for Primate Biology, Davis, CA (Espana, 1971), the Oregon Regional Primate Center (Hall and McNulty, 1967; Nicholas and McNulty, 1968), and the United States Air Force School of Aerospace Medicine, Texas (Casey *et al.*, 1967; Crandell *et al.*, 1969). Between March of 1965 and December 1968, 16 individuals, mainly animal handlers, developed the disease with yaba-like disease virus being isolated from the lesions of three human cases (Espana, 1971). The viral isolates from the three locations have been reported as three separate viruses: yaba-like disease virus (Espana, 1971), Oregon 1211 virus (Nicholas and McNulty, 1968) and yaba-related virus (Crandell *et al.*, 1969). Interestingly, the infected monkeys in the Primate Centers of California, Oregon and Texas had all been supplied by the same importer on whose overcrowded premises African and

Asiatic monkeys had been housed in the same building. Because the disease affected mostly Asiatic monkeys of the genus *Macaca* while African green monkeys were not apparently susceptible, it has been suggested that the virus may have been transmitted to the macaques from asymptomatic African monkeys (Downie and Espana, 1971; Schmidt, 1970).

Morphology of TPV and YLDV Virions

TPV and YLDV have similar morphologies that are distinctive from YMTV (Espana *et al*, 1971). Unlike TPV and YLDV, immature YMTV viruses are spherical in shape and have an electron density only slightly greater than that of the surrounding background (DeHarven and Yohn, 1966).

TPV virions are found to be enveloped at all times during the course of viral replication (Fenner, 1990). Electron microscopic studies on TPV and YLDV virions cultivated in embryonic rhesus monkey kidney (EMK) cells (Espana *et al*, 1971) and CV-1 cells, show biconcave or dumbbell-shaped, DNA containing cores with lateral bodies contained within the concavities. The mature particles also demonstrate a double lipoprotein bilayer that is present on the virions found within both the intra- and extracellular spaces of the EMK cells.

Biological Similarities of TPV and YLDV

TPV and YLDV have been shown to have similar biological properties (Downie *et al.*, 1971; Espana, 1966; Hull, 1968; McNulty *et al.*, 1968). The lesions caused by TPV and YLDV in monkeys were histologically identical and electron microscopic studies have shown the changes produced in infected tissues culture cells by TPV and YLDV to be similar (Espana *et al.*, 1971). TPV and YLDV in CV-1 cell cultures produced small, irregular plaques with dense borders. The density of these borders seemed to result from cell foci formation by irregular retraction from the culture dish. The morphology of both virus-induced cell foci formation was distinctive from that previously demonstrated by Downie and Espana (1973) for YMTV. Early CPE observed at three days post infection, included the development of cytoplasmic vacuoles. Nuclear vacuolization and intense granulation due to the developing viral factories within the cytoplasm were noted at six days post infection (reviewed in Knight *et al.*, 1989). Serological tests such as active immunity experiments, complement-fixation tests, neutralization tests, and precipitation experiments, have failed to demonstrate antigenic differences between TPV and YLDV (Downie and Espana, 1971).

TPV and YLDV Genomes

TPV and YLDV are both double stranded, linear DNA viruses. By calculation of the sum of the DNA digest fragment molecular sizes, the size of TPV and YLDV virion DNA was established to be 145 kbp (Knight *et al.*, 1989). *Pst*I and *Bam*HI restriction endonuclease patterns indicated that TPV and YLDV viruses are closely related; however, they could be discerned as separate strains, quite distinct from YMTV (Knight *et al.*, 1989). While the complete sequence of YLDV has been determined (Lee *et al.*, 2001), only about 17% of the TPV genome has been cloned (Neering, 1993). TPV *Pst*I digested fragments: F (9.0 kbp), H (6.6 kbp), J (5.1 kbp), L (2.1 kbp), M (1.4 kbp), and N (1.1 kbp), were successfully cloned into pUC 19. The *Pst*I-L fragment was completely sequenced, predicted amino acids and putative open reading frames (ORFs) analyzed (Neering, 1993), with the other five fragments having been recently sequenced (unpublished results).

Monoclonal Antibodies

History of Antibody Discovery

Bordet (1895) made an important discovery in 1895 when he found that the clearing of a bacterial infection by a host involved two separable activities. One activity (complement) did not require immunization, but was destroyed by treatment

of serum at 56° C for 30 min. The other required immunization but was stable to heating to 56° C for 30 min. The latter activity was subsequently identified as antibody. Bordet went on to show that lysis of red blood cells by immune serum was functionally equivalent to lysis of bacteria by immune serum and complement (Bordet 1898). Although Bordet was the first to use the term “antibody”, the discovery of antibodies is generally accredited to von Behring and Kitasato (reviewed in Bulloch, 1938; Cohen and Porter, 1964; Silverstein, 1989). In 1890, they showed that immunity to diphtheria and tetanus is due to antibodies to toxins secreted by these organisms.

The knowledge gained about antibodies from Behring and Kitasato was put into clinical use almost immediately following its discovery. In 1891, a child dying of diphtheria was given immune serum and made a recovery that had not been previously reported (Bulloch, 1938). Although the serum sometimes provided dramatic improvements, there were also side effects of the serum administration. Today we know this side effect as anaphylaxis, which is caused by the development of large amounts antigen-antibody complexes throughout the recipient's body.

Brief History of Development of Monoclonal Antibody Technology

In 1975 Kohler and Milstein were the first to describe development of hybrid cells that produced monoclonal antibodies of predefined specificity (Kohler and Milstein, 1975). They described the derivation of a number of tissue culture cell lines

that secreted anti-sheep red blood cell (SRBC) antibodies. The cell lines were made by a fusion of a mouse myeloma with mouse spleen cells taken from an immunized donor.

Each immunoglobulin chain produced from a hybrid cell results from the integrated expression of one of several variable (V) and constant (C) genes coding respectively for its V and C sections from the genome of each parental cell. Each cell expresses only one of the two possible alleles (Milstein and Munro, 1973). When two antibody-producing cells are fused, the products of both parental lines are expressed (Cotton and Milstein, 1973; Schwaber and Cohen, 1974), and although the light and heavy chains of both parental lines are randomly joined, no evidence of scrambling of the V and C sections is observed (Cotton and Milstein, 1973). The results from Kohler and Milstein's work provided the background for the derivation and understanding of antibody-secreting hybrid lines in which one of the parental lines is an antibody-producing spleen cell (Kohler and Milstein, 1975).

Myeloma Cells: Non-Secreting Immortal Fusion Partner

In 1959, it was accidentally discovered that peritoneal irritants could cause the development of myelomas in BALB/c mice (Merwin and Redmon, 1963). A myeloma is the tumor caused by malignantly transformed antibody-secreting cells. Compared with other gene products, there is a rather high frequency of variants and deletions in immunoglobulin synthesis by cultured myeloma cells (Adetugbo *et al.*,

1977) and the loss of heavy chain production is particularly common (Cotton *et al.*, 1973) due to deletions in the V_H gene (Thammana, 1994). The use of myeloma cell lines that produced immunoglobulin product as one of the fusion partners in the production of hybrid cells reduced the quality of the overall yield of the desired antibody (Cotton *et al.*, 1973). This problem was overcome when the clone P3-X63-Ag8.653, which did not express heavy or light chains, was discovered in 1979 (Kearney *et al.*, 1979). The use of this particular clone in fusion experiments allows for the product to be completely of antibody-producing cell origin (Kearney *et al.*, 1979).

Techniques for Cell Fusion and Hybridoma Selection

Electroporation (Steenbakkers *et al.*, 1993; Zimmermann *et al.*, 1990) and electro-acoustic (Bardsley *et al.*, 1990) techniques are alternative techniques that are useful when low numbers of specific B cells are available for fusion. A more traditional method for cell fusion is the treatment of the two cell lines to be fused with either Sendai virus or a high frequency polyethylene glycol (PEG). When cells are treated with either of these two agents, their membranes fuse and multinucleate cells called heterokaryons are formed (Ringertz and Savage, 1976; Abbott and Povey, 1995). At the next cell division, the nuclei of the heterokaryons fuse, and the daughter cells possess a more or less equal share of the genetic material. The exact mechanism of the fusion is still poorly understood (Zimmerberg *et al.*, 1993). An

attempt to understand the mechanism by which PEG promotes cellular fusion (Wojcieszyn *et al.*, 1983) suggests that the coordinate action of two distinct components is necessary for fusion as mediated by PEG. Presumably, the polymer itself promotes close apposition of the adjacent cell membranes, but the additives contained in the commercial PEG provide the fusion stimulus.

When a cell mixture, cell A/cell B, is subjected to reagents that promote fusion there are three possible hybrid cells that can be formed: A-A, A-B and B-B. Thus, if it is desired to produce the A-B fusion as a long-term hybrid cell line from two cells, a selection procedure is required. The most common selection procedure is that devised by Littlefield (1964). Briefly, this selection procedure depends on the fact that when the main biosynthetic pathway for guanosine is blocked by the folic acid antagonist aminopterin, there is an alternative "salvage" pathway in which the nucleotide metabolites hypoxanthine or guanine are converted to guanosine monophosphate via the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). Cells lacking HPRT die in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium) because both the main and salvage pathways are blocked. However, an HPRT- (myeloma) cell can be made to grow in HAT medium if it is provided with the missing enzyme by fusion with an HPRT+ cell (B-cell from an immunized donor).

Lyon (1964) and Littlefield (1964) found, due to the fact that myeloma cells will spontaneously mutate to form HPRT+ cells, it is essential to select for the proper myeloma fusion partner prior to cellular fusion by selection of HPRT- myeloma cells

with the use of the toxic base analogue 8-azaguanine, which is incorporated into the DNA via HPRT. Because the salvage pathway is not normally essential for cell survival, mutants that lack HPRT will continue growing, while cells that possess HPRT will die.

Applications of Monoclonal Antibodies

Since the development of the technique by Kohler and Milstein in 1975, there have been many monoclonal antibodies developed by a vast number of different investigators to assist in their studies and diagnoses of novel systems and chronic diseases. This section will outline only a few in order to provide a better understanding of the many utilizations that monoclonal antibodies can provide and lend to the reader an appreciation of the technique that resulted in Kohler, Milstein and Jerne being awarded the Nobel Prize in Immunology in 1984.

Monoclonal antibodies are useful research tools in the analyses of structure and function of microbial and other proteins, and have become powerful reagents in the diagnosis and treatment of infection and biomedical research. Antibody-based specific identification and detection of microorganisms, including viruses, and their respective antigens form the basis of a large array of techniques that are indispensable to almost all areas of basic research and are becoming more widely used.

The power that is provided by antibody-based techniques can be increased with the use of monoclonal antibodies as opposed to polyclonal antisera. Monoclonal

antibodies have not completely replaced polyclonal antisera in many applications of the antibody-based technique, but their availability has greatly expanded the scope of antibody usage.

An example of the superiority of monoclonal antibodies over polyclonal antisera is in the analysis of functional membrane proteins such as cytokine receptors, adhesion molecules, signal transduction and enzymes. Monoclonal antibodies developed against human leukocyte differentiation antigens (Knapp *et al.*, 1989; McMichael *et al.*, 1987; Schlossman *et al.*, 1994) have been used to identify and characterize more than at least 100 new molecules, which were previously unknown.

Monoclonal antibodies can also be used to stabilize molecules by forming antigen-antibody complexes that can then be used for structural studies. Moore *et al.*, (1993) used monoclonal antibodies to resolve the structure of the viral surface envelope glycoprotein gp120 of human immunodeficiency virus (HIV).

Grose *et al.* (1983) used the technique to develop monoclonal antibodies against the varicella-zoster virus (VZV). They describe three prominent glycoproteins- gp62, gp98 and gp118, which are coded for by VZV from infected cells. They demonstrated the development of four monoclonal antibodies, which reacted with both gp62 and gp98, and one that reacted with gp118. Also, when assayed for neutralizing activity, the secretory product of the single anti-gp118 hybridoma, but not the supernatants from the four anti-gp-62/98 clones, inhibited VZV plaque formation by greater than 80%. Thus, Grose *et al.* (1983) used a

monoclonal antibody to show that at least one of the glycosylated antigens of VZV is a determinant that elicits neutralizing activity of VZV infection.

Tang *et al.* (2001) have reported the development of a new treble-coated enzyme-linked immunoadsorbent assay (ELISA) kit for detecting hepatitis B virus (HBV) surface antigen subtypes a, d and r (HbsAg-a, -d, -r) through the establishment of four hybridoma cell lines. Their results indicated that this new kit was more rapid and sensitive than other current ELISA-based kits coated with a single monoclonal antibody (e.g., anti-HbsAg-a). These results show that a series of monoclonal antibodies, targeting different antigens on the same pathogen, can be used in conjunction with one another to increase the accuracy of results gathered from such tests as ELISAs.

Aside from the role that monoclonal antibodies can play in the analysis and detection of viral pathogens, they can also be used in the analysis of signal transduction. Houlden *et al.* (1991) have generated an anti-mouse class I reactive monoclonal antibody that is capable of activating T cells in the presence of cofactor PMA, as assayed by both interferon- γ production and cellular proliferation. This monoclonal antibody also recognizes multiple human class I molecules. The data gathered by the investigators have demonstrated the potential for the involvement of class I molecules, which are usually not considered as activation antigens, in the signal transduction pathway ultimately leading to the activation of T cells in the immune response.

Monoclonal antibodies have also proven to be a useful tool in the study of organ system structure and function. Quaroni (1983) used Kohler and Milstein's technique to develop nineteen monoclonal antibodies directed against intestinal brush boarder membrane proteins. Ten of the antibodies were shown to specifically immunoprecipitate surface-membrane proteins. Two antibodies were found to be specific for sucrase-isomaltase, one for an aminopeptidase, two for an isoenzyme of alkaline phosphatase that is exclusively in the proximal small intestine, and one for maltasae-glucoamylase. These monoclonal antibodies should prove to be invaluable tools for the study of the biosynthesis of cell-surface proteins, the fetal and postnatal development of specific intestinal functions, and the process of cell differentiation in the intestinal epithelium.

The use of monoclonal antibodies directed against cancer cells has found increasing usefulness in recent years. Relative tumor specificity and a lack of significant toxicity, together with the ability to link radionuclides without significant deterioration of biological behavioral characteristics such as immunoreactivity, have enabled widespread use of radiolabeled monoclonal in several malignancies, including malignant melanoma and breast cancer (Divgi and Larson, 1989; Merno, Monteagudo, and Neumann, 1991).

Conclusions

Having demonstrated the biologic and genomic similarities demonstrated between TPV and YLDV, also called Oregon 1211 virus and yaba-related virus, coupled with the fact that the monkeys from which YLDV was isolated from outbreaks in the primate center of Oregon, Texas, and California were received from the same importer, it stands to reason that these are simply different strains of the same virus. The nomenclature of Tanapox virus- Kenya strain and Tanapox virus- OrTeCa strain is being suggested by our laboratory as names that would simplify the classification of this virus.

Given the power of the hybridoma technique as an experimental approach for the analysis of complex biological systems, it was the intent of this project to develop a bank of monoclonal antibodies that could be used to demonstrate the conservation of individual antigens of both TPV and YLDV in an effort to further identify these viruses as different strains of the same virus. Also, since TPV and YLDV are related to smallpox virus, but with greatly reduced virulence, a bank of monoclonal antibodies directed against these viruses could prove to an invaluable tool in the study of these viruses as models of the virus-host interaction.

MATERIALS AND METHODS

Virus, Cells and Reagents for Virus Cultivation

Owl monkey kidney (OMK) cells (American Type Culture Collection, Rockville, MD) were grown in RPMI 1640 (Life Technologies, Grand Island, NY) supplemented with 10.0 % (v/v) newborn calf serum (NBCS) (Life Technologies), 2.0 mM L-Glutamine, and antibiotics (100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulphate and 0.25 µg/ml amphotericin B) (Life Technologies)[OMK growth medium]. OMK cells were maintained in RPMI 1640 supplemented with 2.0 % (v/v) NBCS, 2.0 mM L-Glutamine, and antibiotics (maintenance medium). Yaba-like disease virus (YLDV) (American Type Culture Collection) was cultivated in OMK cell monolayers maintained in maintenance medium at 37° C with 5 % CO₂.

Virus Concentration

Confluent Monolayers of OMK cells were infected with YLDV at 1.0-2.0 plaque forming units per cell (pfu/cell). Following adsorption for 1.0 hour on a rocker table at room temperature (RT), infected cells were maintained in maintenance medium and incubated for 10-14 days. Infected cells were harvested using a sterile

cell scraper (S/P[®]Brand dispo[®] Cell Scraper, Baxter, McGraw Park, IL) and collected as 1X virus mixture. The 1X virus mixture was then centrifuged (approximately 100 x g, Damon IEC PR-6000 at 2000 rpm) at 4.0° C for 20.0 min. The supernatant from this centrifugation was then saved and each cell pellet was then resuspended in 1.0 ml maintenance medium and subjected to three cycles of freeze/thaw (ethanol and CO₂(s) mixture was used to freeze with 37.0° C H₂O bath used to thaw) to release all intracellular virus particles. OMK cellular debris was removed from each sample by centrifugation (100 x g, Damon IEC PR-6000, at 2000 rpm) at 4.0°C for 20.0 min. The supernatant from these samples and that saved from the previous centrifugation of the 1X virus mixture was then centrifuged at 85,000 x g, (Beckman Optima XL-100K ultracentrifuge; Ti-45 rotor at 30,000 rpm) for 1.5 hours at 4.0° C. Each virus pellet was then resuspended overnight in maintenance medium to a final concentration on 100X and quantified by viral plaque assay (Essani, 1982). Briefly, OMK cells monolayers grown in 6-well plates were infected with 200.0 µl/well of serial ten-fold dilutions of 100X YLDV, with each dilution being plated in duplicate. Virus was adsorbed at RT for 1.0 hour on a rocker table. Following adsorption, 2.5 ml of overlay medium [maintenance medium with 0.5 % methyl cellulose (4000 centipoises) (Fisher Scientific, Pittsburgh, PA)] was added to each well and the plates were then incubated at 37.0° C with 5.0 % CO₂ for 10-14 days. Medium was then carefully aspirated from each well, without disturbance of the monolayer, and 1.0 ml of 0.1 % crystal violet and 10.0 % formaldehyde in deionized water was then added to each well to fix and stain the cells for 30.0 min at RT. Plates were then rinsed with

distilled water and allowed to dry. Plaques were counted to determine pfu/ml by the following formula: $\text{pfu} = ((\text{number of plaques in well-A} + \text{plaques in well-B}) / 2) \times \text{dilution factor for that duplicate of wells}$, then $[\text{number pfu (200.0 } \mu\text{l)}] \times 5 = 1.0 \text{ ml pfu}$.

Immunization

Authorization for the use of an animal model for this research study was obtained under IACUC protocol number 95-08-01. Under this protocol, no adjuvant was used to enhance the immune response. Accordingly, female, BALB/c mice, 4-6 weeks old (Charles River Laboratories, Portage, MI) were immunized with YLDV through a series of five weekly 100.0 μl , intraperitoneal (IP) injections of 100X YLDV. The final or booster injection was administered four days prior to the fusion. Each mouse was monitored daily for any complications due to the immunization protocol.

Fusion

Myeloma Cells and Reagents

Mouse plasmacytoma, P3x63.Ag8.653 (American Type Culture Collection) (myeloma cells) cells were grown in RPMI 1640 supplemented with 20.0 % (v/v)

HY-CLONE, fetal bovine serum, (FBS) (Sterile Systems, Inc, Logan, UT), 2.0 mM L-Glutamine, and antibiotics (hybridoma growth medium) at 37.0° C with 5.0 % CO₂. Two weeks prior to the fusion date, the myeloma cells were grown in myeloma growth medium supplemented with 6.6×10^{-2} M 8-Azaguanine (Sigma, St. Louis, MS) for selection of HPRT (-) (Littlefield, 1964) cells for one week, about 3-4 growth cycles. Following this one-week treatment with 8-Azaguanine, the myeloma cells were once again grown in myeloma growth medium without 8-Azaguanine for one week prior to the fusion date. The cells were fed one day prior to the fusion to ensure that the cells were in the exponential growth phase for the fusion.

Myeloma Cell Quantification

The P3x63.Ag8.653 cells were counted using a hemocytometer in standard fashion to ensure the correct ratio of immunized splenocytes to myeloma cells, 10:1 respectively (Zola, 1995). Briefly, the cells were maintained in a 75 cm² tissue culture flask, kept upright, containing 5.0 ml/flask of hybridoma growth medium. The flask was then gently shaken to resuspend all cells and a sterile, glass, pasture pipette was then dipped into the solution with the forefinger being placed over the end of the pipette following emersion of the pipette tip to create a vacuum, as to keep solution within the pipette. A glass cover slip was then placed of the counting grid of the hemocytometer and the pipette was placed along the edge of the cover slip to allow diffusion of the solution under the slip and subsequently over the counting grid. The

counting grid consists of four equally partitioned square areas separated into quadrants by a series of lines within the overall grid area. All individual cells, not clusters, that lie on the upper and right boundaries of each individual partition are counted to calculate a sum for each of the four square areas. The following formula is then used to calculate the total number of cells/flask:

$\{[(\text{sum quad 1})+(\text{sum quad 2})+(\text{sum quad 3})+(\text{sum quad 4})]/4\} = \text{avg. cell number/quad}$; $(\text{avg. cell number/quad}) \times 10^4 = \text{number of cells/ml}$; $[(\text{number of cells/ml}) \times (\text{total volume per flask})] = \text{total number of cells/flask}$.

“Feeder Cells”

A modification to the original procedure (Fazekas and Scheidegger, 1980) for the development of “feeder cells” was used. “Feeder cells” were obtained from a non-immunized, female, BALB/c mouse 24.0 hours prior to the fusion. A 1.0 ml injection of sterile, phosphate buffered saline (PBS) (137.0 mM NaCl, 2.7 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 ; pH 7.4) was administered intraperitoneally to the mouse to be used for the harvesting of the “feeder cells”. Following the injection of PBS, the abdomen was massaged to wash the peritoneal cavity. The mouse was sacrificed by cerebral dislocation and the skin of the abdomen was retracted exposing the peritoneum. A sterile, glass, pasture pipette, plugged with cotton, was then used to remove the PBS from the peritoneal cavity. The PBS was then added to 50.0 ml conical centrifuge tube containing 49.0 ml hybridoma growth medium, with this

solution then used to perform five subsequent washes of the peritoneal cavity. The centrifuge tube was then vortexed and its contents poured into a sterile petri dish. An eight-channel pipette (Titertek® digital multichannel pipette 50-200µl, Flow Laboratories, Finland) was then used to distribute 100.0 µl into each well in five separate polystyrene, flat-bottom, 96-well tissue culture plates (Corning Glass Works, Corning, NY). The “feeder cells” were then incubated overnight at 37.0° C with 5.0 % CO₂.

Fusion Protocol

Modifications to the original fusion protocol (Kohler and Milstein, 1975) were made and the procedure completed as outlined. An immunized BALB/c mouse was sacrificed by cerebral dislocation and soaked in 70.0 % ethanol for 5.0 min. A 4.0 cm incision was then made along the centerline of the abdomen exposing the peritoneal cavity. The spleen was then located and a splenectomy performed by cutting the Splenorenal ligament, Splenic artery, Splenic vein and all connective tissue. The excised spleen was then transferred into a 50.0 ml conical centrifuge tube containing 5.0 ml of RPIM 1640 supplemented with antibiotics only (serum-free medium) at 37.0° C. The spleen was then placed on a sterile, stainless steel mesh covering a 400ml sterile, glass beaker. A rubber policeman was then used to homogenize the spleen to separate all splenocytes from residual fat and fascia. The stainless steel mesh was then washed with an additional 25.0 ml serum-free medium at 37.0° C.

The contents of the beaker (serum-free medium and immunized splenocytes) along with a flask of myeloma cells (prepared as previously discussed) were added to a 50.0 ml conical centrifuge tube and centrifuged (75 x g, Damon IEC HN-SII, at 1500 rpm) at RT for 10.0 min. The supernatant was then removed and 25.0 ml serum-free medium at 37.0° C was then added to resuspend the pellet through gentle inversion. The contents of the tube were once again centrifuged (75 x g, Damon IEC HN-SII, at 1500 rpm) at RT for 10.0 min. This cycle was repeated one more time for a total of three wash cycles. Following the third wash of the immunized splenocyte/myeloma cell pellet, 100.0 µl of serum-free medium was left in the centrifuge tube to resuspend the pellet. A volume of 1.0 ml of polyethylene glycol (PEG) 4000 (Life Technologies) at 37.0° C was then added to the resuspended cells over the course of 1.0 min. A slow dilution of the PEG was then carried out with serum-free medium over the next 21.0 min as follows: 10.0 ml over 10.0 min; 10.0 ml over 5.0 min; 10.0 ml over 3.0 min; 10.0 ml over 2.0 min; and 9.0 ml over the last 1.0 min, for a total dilution of 49:1, serum-free medium to PEG respectively. This mixture was then centrifuged (75 x g, Damon IEC HN-SII, at 1500 rpm) at RT for 10.0 min. The resultant supernatant was then discarded and the pellet of fused cells was then resuspended in 50.0 ml of hybridoma growth medium. The fused cellular suspension was then poured into a sterile petri dish. At this time, the 96-well dishes containing the “feeder cells” were removed from the incubator and the medium aspirated from each well. An eight-channel pipette was then used to distribute 100.0 µl of the suspension to each well. The 96-well dishes were then incubated for 24.0

hours at 37.0° C with 5.0 % CO₂. Following 24.0 hours, an additional 100.0 µl of hybridoma growth medium supplemented with 8x10⁻⁷ M aminopterin, 3.2x10⁻⁵ M thymidine, and 2x10⁻⁴ M hypoxanthine (HAT) (Life Technologies) (2X selection medium) was added to each well (Littlefield, 1964). All subsequent medium changes that were required for each individual well, within the first three weeks following the fusion procedure, were completed by removal of 100.0 µl of well supernatant followed by the addition of 100.0 µl of hybridoma growth medium supplanted with 4x10⁻⁷ M aminopterin, 1.6x10⁻⁵ M thymidine, and 1x10⁻⁴ M hypoxanthine (HAT) (selection medium). All medium changes within wells following the first three weeks were completed with hybridoma growth medium.

Hybridoma Screening Assay

Collection of Hybridoma Supernatant

All wells were observed daily for hybridoma colony formation. Once a viable colony had been discovered, it was monitored daily for necessary medium changes; medium was considered to be depleted of nutrients upon color change from red to orange/yellow of the indicator- Phenol Red contained within the RPMI 1640. Upon color change within any given well, 100.0 µl of the well's supernatant was removed and stored at 4.0° C with 100.0 µl of either selection medium or hybridoma growth

medium, dependent upon the time that past following the fusion (see above- fusion protocol), being added the well.

Development of Cover Slips

OMK cell monolayers were grown on 25 cm², glass cover slips (Corning), sterilized by autoclaving, in 100 cm² tissue culture dishes in OMK growth medium. OMK cells were then infected at 50.0 pfu/cell with YLDV in standard fashion (see above- virus concentration) and maintained in maintenance medium until 80-90 % of all cells demonstrated cytopathic effect (CPE), 5-7 days post infection. Maintenance medium was then aspirated and 100.0 % ethanol at -80.0° C was then added to each plate for 30.0 sec for cell fixation. Following fixation, the ethanol was removed and the fixed cover slips were stored in PBS at 4.0° C. Mock cover slips were fixed in identical fashion following the development of confluent monolayer and mock infection with maintenance medium for 5-7 days.

Indirect Immunofluorescence (IF) Assay

A hybridoma colony was screened for anti-YLDV antibody production once the colony had reached 50-70 % confluence within the well it was contained. Supernatant was removed from the well in 100.0 µl quantities to provide adequate volume for screening against both uninfected and YLDV infected OMK cells. Cover

slips were first washed three times for 5.0 min intervals at RT with PBS with 0.1 % gelatin (BioRad, Richmond, CA) pH 7.3, to block any non-specific protein binding. Each cover slip, both an uninfected and infected, was covered with 50.0 μ l of the hybridoma of interest's supernatant and incubated at 37.0° C in a humidified incubator for 2.0 hours. Each cover slip was then washed three times for 5.0 min intervals at RT with PBS, pH 7.3 to remove all non-bound protein. Following this washing cycle 50.0 μ l of an anti-mouse IgG (whole molecule) Fluorescein Isothiocyanate (FITC) conjugate (anti-mouse IgG-FITC) developed in goat (Sigma), diluted 1:100 in PBS with 0.1 % EIA grade gelatin (BioRad), pH 7.3 was then added to each cover slip followed by an additional two hour incubation period at 37.0° C in a humidified incubator. Each cover slip was again washed three times for 5.0 min intervals at RT with PBS, pH 7.3 to remove all non-bound secondary antibody. Each cover slip was then mounted on a glass slide (Sigma) with 20.0 % glycerol in PBS, pH 7.3 and sealed with "Built-in Topcoat" (Cover Girl, Hunt Valley, MD). Slides were then viewed on a Nikon MICROPHOT-FXA (Nikon, Tokyo, Japan), with filter set: excitation filter 450-490 nm and barrier filter 520 nm, using a super high pressure mercury lamp (Model HB-10101AF, Nikon) for positive staining results.

Maintenance of Positive Clones

Cloning by Limiting Dilution

Slight modifications to the original procedure (Lefkovits and Wadlmann, 1979) were implemented. All hybridoma clones that produced positive IF results were cloned to ensure the presence of only one producing clone. Positive clones were suspended through pipetting and plated out in serial twofold dilutions in 200.0 μ l cultures in 96-well dishes (Corning). The group in which about half of the wells had no growth was considered to contain the progeny of single cells (Goding, 1996). This group was then rescreened through IF for antibody production. All hybridoma clones that produced a second positive IF result were then recloned through the same procedure for cloning by limited dilution. Colonies were allowed to grow in 200.0 μ l of myeloma growth medium until 70 % confluence was achieved within the well. The cells of four separate wells from a 96-well dish, displaying 70 % confluence, were then resuspended and transferred to a 24-well dish (Corning) and grown in 1.0 ml hybridoma growth medium. Again, once 70 % confluence was achieved in these wells, the cells from two of these wells resuspended and transferred to a 12-well dish (Corning) and grown in 2.0 ml of hybridoma growth medium. Finally, the cells from an individual well in a 12-well dish at 70 % confluence were then transferred to a 75 cm^2 tissue culture flask and grown in total volume of 5.0 ml hybridoma growth medium.

Cryopreservation of Hybridoma Cells

Positive hybridoma cells were grown in suspension culture in 75 cm² tissue culture flasks in 5.0 ml of hybridoma growth medium. Each flask to be frozen was fed the day before the freezing procedure to ensure that the cells were in the exponential growth phase; 2.5 ml of the supernatant was removed and 2.5 ml of fresh hybridoma growth medium at 37.0° C was added. The flask was then incubated for 24.0 hours at 37.0° C with 5.0 % CO₂. The cells were then resuspended in the flask by pipetting and transferred to a 15.0 ml conical centrifuge tube and centrifuged (75 x g, Damon IEC HN-SII, 1500 rpm) at RT for 10.0 min. The supernatant was then removed and the cells were resuspended in 1.0 ml of hybridoma growth medium supplemented with 10.0 % sterile dimethyl sulfoxide (DMSO) (freezing medium). The cells were then transferred to a 1.2 ml freezing vial (Gibco, Grand Island, NY), placed in a freezing container (Cryo 1 C Freezing Container, Nalgene, Rochester, NY), to achieve a -1.0° C/min rate of cooling, and stored in a -80.0° C freezer for 24.0 hours. Following this 24.0 hour period, the cells were then immediately transferred into holding racks and immersed in N₂ for preservation.

Test of Cryopreserved Hybridoma Viability

Three days following initial cryopreservation, a freezing vial containing the hybridoma of interest was removed from N₂ (l) and immediately transferred to a 37.0°

C water bath and thawed for 3.0 min. The contents of the tube were then diluted in 19.0 ml hybridoma growth medium at 37.0° C in 75 cm² tissue culture flask and incubated at 37.0° C with 5.0 % CO₂ for 48.0 hours. The viability of hybridoma cells was then visually assed following the 48.0 hour period. The supernatant from hybridoma clones thawed following cryopreservation was also tested for antibody production through immunofluorescence and immunodiffusion.

Hybridoma Characterization

Hybridoma Supernatant Concentration

Supernatant from hybridoma clones that were found to give initial positive IF results was concentrated 25X using a Minicon® B-15 Clinical Sample Concentrator (Millipore, Bedford, MA) thorough standard technique. Briefly, each sample was prefiltered through Whatman No. 1 filter paper. A glass pasture pipette was then used introduce the sample into the sample well and allowed to adsorb overnight. Each sample well was able to concentrate ~5.0 ml hybridoma supernatant to a final concentration of ~25X. Following, overnight adsorption, each sample was removed and stored at 4.0° C for later use in characterization experiments.

Indirect Immunofluorescence (IF) Assay and Digital Imaging

All hybridoma clones that were found to have initial positive staining results were then taken through IF assays in triplicate. Three sets of cover slips were set for each clone as follows: 1) uninfected cells treated with hybridoma supernatant as primary antibody and anti-mouse IgG-FITC as secondary antibody; 2) infected cells treated with hybridoma supernatant as primary antibody and anti-mouse IgG-FITC as secondary antibody; and 3) infected cells treated with anti-mouse IgG-FITC only. MetaMorph imaging software- version 4.1.7 (Universal Imaging Corporation, Downingtown, PA) together with a Nikon MICROPHOT-FXA (Nikon, Tokyo, Japan) was then utilized to obtain digital pictures of all slides through IF, using a super high pressure mercury lamp (Model HB-10101AF, Nikon), and differential interference contrast (DIC) microscopy.

Hybridoma Isotype Determination- Immunodiffusion (ID) Assay

Ouchterlony double diffusion technique was used to determine the heavy chain isotype of all antibodies produced by a hybridoma producing positive IF results. A solution 1.0 % (w/v) agar and 0.01% (w/v) sodium azide in deionized water was heated to 95.0° C in microwave oven, plated on a glass slide and allowed to cool and form an agar slab. An outline was then determined to have six outer wells surrounding one center well all equal distance- ~1.5 cm. A mouse monoclonal

antibody isotyping kit (Sigma) was then used per protocol. Briefly, 10.0 µl of the concentrated hybridoma supernatant was added to the center well with 5.0 µl of each of the isotyping reagents- anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG3, anti-mouse IgA, and anti-mouse IgM; being added to the outer wells. The slide was then incubated at 37.0° C in a humidified incubator for 24.0 hours to allow for diffusion of all reagents. Following incubation, the slide was removed from the incubator and placed in PBS at RT for 24.0 hours at RT to elute all non-conjugated protein. The slide was then covered with a moist paper towel and allowed to dry for an additional 24.0 hours at RT. Once the agar had completely dried, an agar staining solution (0.1% Coomassie Brilliant Blue in deionized water) was added to the slide for 5.0 min for protein staining. Following staining, the slide was rinsed with deionized water and placed in destaining solution (5.0 % methanol, 7.0 % glacial acetic acid in deionized water) for 10.0-15.0 hours at RT. The slide was then removed from the destaining solution and allowed to air dry.

Immunoprecipitation of Viral Antigen by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Preparation of Radiolabeled Viral Antigen

OMK cell monolayers- consisting of 5.2×10^5 cells (Mediratta, 1997), grown in 60 mm² tissue culture dishes (Corning), were infected with 200.0 µl of 100X YLDV at a multiplicity of infection of ~50.0 pfu/cell until 80-90 % of all cells displayed

CPE, 5-7 days post-infection. A mock set OMK cell monolayers in 60 mm² dishes was also maintained in maintenance medium for 5-7 days. Once 80-90 % of all infected cells displayed CPE, the medium was aspirated from each dish and replaced with maintenance medium containing 12.5 µCi/ml [¹⁴C]-L-amino acid mixture (specific activity 50.0 mCi/mmol per carbon atom; ICN Biomedicals, Inc, Irvine, CA) and incubated for 18.0 hours at 37.0° C with 5.0 % CO₂. Following incubation, cells were scrapped using a sterile cell scrapper (S/P[®]Brand dispo[®] Cell Scraper, Baxter, McGraw Park, IL), transferred into a 15.0 ml conical centrifuge tube and centrifuged (75 x g, Damon IEC HN-SII at 1500 rpm) at RT for 10.0 min. Supernatant was collected and stored at 4.0° C. The cell pellets were then resuspended in 100.0 µl of 1.0 % (v/v) Nonidet P40 (NP-40) for 5.0 min on ice to lyse plasma membrane. Low speed centrifugation (approximately 15 x g, Damon IEC HN-SII at 300 rpm) was then carried out for 5.0 min to remove nuclei. A 95.0 µl volume of the supernatant was then removed as not to resuspend the nuclear pellet. A 5.0 µl aliquot was then added to 25.0 µl of dissociation buffer (DB) [0.0625 M Tris-HCl (pH 6.8), 2.3 % (w/v) sodium dodecyl sulphate (SDS), 10.0 % (w/v) glycerol, 5.0 % (v/v) 2-mercaptoethanol (ME) and 0.001% bromophenol blue] for running of whole protein preparation on gel. A 2X Immunoprecipitation buffer (IPB) (20 mM Na₂PO₄ pH 7.4, 0.3 M NaCl, 2.0 % deoxycholate, 2.0 % triton X-100) was added to the supernatant in a 1:1, (v:v) ratio of NP-40: IPB to give the final immunoprecipitation solution (IPS) . The samples were then solubilized in a water bath at 37.0° C for 30.0 min. Following heating, samples were prepared, in duplicate, for immunoprecipitation as follows: 1)

45.0 µl IPS with 45.0 µl 25X hybridoma supernatant; 2) 50.0 µl IPS with 50.0 µl of a 1:100 dilution of anti-mouse IgG2 antibody isotype reagent (anti-mouse IgG2) (Sigma), to control for non-specific binding to antibody molecules. These samples were then allowed to immunoprecipitate for 24.0 hours in a 37.0° C water bath. After 24.0 hours, 20.0 µl of a 1:50 dilution of an anti-mouse IgG (whole molecule) (Sigma) was added to one of the replicates from each of the two sets of immunoprecipitation samples, with the other replicate receiving the addition of 20.0 µl Protein A/G PLUS-Agarose: sc-2003, prepared per protocol, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and incubated in a water bath at 37.0° C for 4.0 hours. The samples were then centrifuged (Brinkman 5415C, at 12,000 x g) for 20.0 min at RT. The supernatant was removed and stored at 4.0° C. The pellet was then washed three times with 0.85 % (w/v) NaCl in deionized water with subsequent centrifugation (Brinkman 5415C, at 12,000 x g) for 20.0 min between each wash. Each pellet was then resuspended in 30.0 µl of DB and heated to 100.0° C for 3.0 min for loading onto SDS-PAGE gel. Mock samples were prepared in identical fashion of YLDV infected samples.

SDS-PAGE Resolution of Viral Antigen

Radiolabeled immunoprecipitates from both YLDV infected and mock OMK cells were resolved by electrophoresis on an 11.5 % polyacrylamide gel [29.2 % (w/v) acrylamide: 0.8 % (w/v) bisacrylamide] as described by Essani and Dales

(1979). Briefly, the upper or stacking gel consisted of consisted of 6.0 ml distilled water, 2.5 ml upper gel buffer (0.5 M Tris-Base, 0.4 % (w/v) SDS, pH 6.8), 1.5 ml of 30.0 % acrylamide (29.2 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide), 30.0 μ l of 10.0 % (w/v) ammonium persulphate and 10.0 μ l of TEMED (N, N, N', N' tetra methyl ethylene). The lower or separating gel consisted of 10.7 ml deionized water, 7.5 ml lower gel buffer (1.5 M Tris-Base, 0.4 % (w/v) SDS, pH 8.8), 11.5 ml 30.0 % acrylamide, 300.0 μ l of 10.0 % (w/v) ammonium persulphate and 15.0 μ l of TEMED. The lower gel was poured and allowed to polymerize for 1.0 hour. A comb was then inserted and the upper gel poured around the comb and allowed to polymerize to form the sample wells of the gel. Samples, including a high molecular weight standard (BioRad), were prepared (see above- preparation of radiolabled antigen) and loaded onto the gel using a 100.0 μ l, glass microsyringe and electrophoresed at 50.0 mA in 1X running buffer (0.025 M Tris-Base, 0.192 M glycine, and 0.1 % (w/v) SDS) for 4.0 hours. Following electrophoresis, the gel was placed in a staining solution (0.25 % Coomassie Brilliant Blue, 50.0 % methanol, and 10.0 % acetic acid) at RT for 18-24 hours. The gel was fixed and destained in destaining solution (5.0 % methanol, 7.5 % glacial acetic acid in deionized water) at RT for 7-10 hours on a rocker table. Following destaining, the gel was washed with deionized water, placed on a Whatmann 3 MM paper cut to size, and covered on the exposed side with clear plastic wrap. The gel was then dried on a slab drier (Model 443 Slab Dryer, BioRad) at 80.0° C with vacuum for 3.0 hours. The dried gel was then wrapped in clear plastic wrap and exposed to Kodak X-OMAT AR film (Eastman Kodak Company,

Rochester, NY) for 5-7 days. The film was then developed at RT by placement in developer (Kodak) for 5.0 min, stop solution (5.0 % (v/v) glacial acetic acid in deionized water) for 2.0 min and fixer (Kodak) for 5.0 min. The film was washed for 30.0 min with deionized water and then allowed to air dry at RT.

Hybridoma Cross Reactivity Assay

Determination of cross reactivity of hybridoma supernatants with TPV (Centers for Disease Control) was achieved through modifications of the procedure outlined for IF screening (see above- Indirect Immunofluorescence and Digital Imaging). Briefly, a triplicate set of OMK cell monolayers grown on cover slips (Corning) were infected with the TPV isolate from the Centers for Disease Control (CDC). The screening assay was then completed identically to the aforementioned IF assay for determination of hybridoma production of anti-YLDV (ATCC) antibodies.

Determination of Viral Antigen as Either Early or Late Protein

Modifications to the IF screening assay were used to determine if the viral antigen, to which any developed antibody produced was directed, is either an early or late protein in the YLDV DNA synthesis pathway. OMK cell monolayers were grown on cover slips as previously discussed (see Indirect Immunofluorescence). The OMK cells were infected with YLDV in similar fashion and the virus particles

allowed to absorb for 1.0 hour at RT on a rocker table. Following adsorption, the supernatant was removed from the dish and 2.0 ml of maintenance medium supplemented with 40.0 µg/ml of cytosine arabinoside (Ara-C) was added and the dish incubated at 37.0° C with 5.0 % CO₂ for 5-7 days; time course determined by CPE displayed in dishes infected with YLDV maintained in maintenance medium without addition of Ara-C- mock infected dishes. The supernatant was then removed from the dish and the cells fixed per IF protocol. The cover slips were then taken through the IF screening assay per protocol and viewed for positive staining results.

RESULTS

Development of Monoclonal Antibodies Directed Against Yaba-Like Disease Virus

Modifications to the technique developed by Kohler and Milstein (1975) were used to develop hybridoma clones that produced monoclonal antibodies directed against YLDV. Female BALB/c mice, 4-6 weeks old, were immunized against YLDV, the immunized B-cells were then isolated and fused with mouse myeloma cells via PEG. Over the course of two and one-half years, seven fusions were carried out in our laboratory with varying results. The first fusion experiment resulted in 130 wells containing viable clones. All of these clones tested negative for anti-YLDV antibody production through an IF screening assay (Table 1). The second and third fusions both resulted in similar numbers of clones surviving to a point at which they could be screened, all resulting with negative IF results.

Additional modifications to the procedure, as well as the materials used, were made following the third fusion to possibly increase the number of viable clones that were produced. One of the modifications was reducing the centrifugation speed from 1500 rpm to 1000 rpm used to pellet fused cells directly following dilution of the PEG. This was done to possibly reduce the lysis of the freshly fused cells due to forces exerted on the plasma membranes during centrifugation. The water used for the preparation of all media for the hybridoma cells was changed from deionized

water obtained from the departmental common facility, to sterile water for irrigation (Baxter Healthcare Corporation, Deerfield, IL), to possibly eliminate any residual endotoxin from the water source. Finally, the lot of FBS to be used for the media was selected based on visual assessment of myeloma cell quality maintained in medium supplemented with three separate lots, with the lot resulting in the highest number of viable cells per flask being selected.

Table 1

Summary of Number of Wells Screened per Fusion with Immunofluorescence Results

Fusion number	Wells Screened ^a	Negative IF ^b	Positive IF ^c
1	130	130	0
2	187	187	0
3	224	224	0
4	384	384	0
5	282	282	0
6	414	414	0
7	372	371	1

a = 100.0 μ l well supernatant was removed screened against YLDV infected and mock infected OMK cells through IF; b = staining patterns similar on both YLDV infected and uninfected cells; c = staining pattern resulting in high intensity fluorescence on infected cells as compared to uninfected cells.

These procedural modifications resulted in a greater number of viable cells, but no increase in the number of clones. From all fusions only one hybridoma clone-H12, produced positive results through IF when supernatant was assayed against YLDV-infected OMK cells and uninfected OMK cells.

The H12 clone developed in row H, column 12 of the third plate used in the seventh fusion. H12 was recloned through limiting dilution and rescreened via IF. Again, the supernatant from wells containing the recloned H12 cells produced positive IF results against YLDV-infected cells and negative IF results against uninfected cells. H12 was then cultivated and ultimately grown in 75 cm² tissue culture flasks for the production of large amounts of supernatant. H12 cells were cryopreserved and three vials removed for testing of viability and antibody production. All three vials removed from N₂ produced viable cells and supernatant that resulted in positive IF screening against YLDV-infected cells and negative IF screening against uninfected cells.

Hybridoma Clone H12 Produces an IgG1 Isotype Antibody

The technique developed by Oudin and Ouchterlony (reviewed in Nilsson, 1967) takes advantage of the cross-linking between an antibody contained in an antiserum and a large antigen such as a protein or a polysaccharide. The cross-linking of these molecules often results in the formation of a precipitate. In this

technique the antigen and antibody are allowed to diffuse towards each other through agar, the precipitate will then be in the form of a visible line.

This technique was utilized to determine the isotype of the antibody secreted by the H12 hybridoma clone. The supernatant from H12 cells grown in a 75 cm² tissue culture flask was concentrated 25X in a B-15 clinical sample concentrator (Millipore). The concentrated supernatant was then placed in a center well cut in a solidified agar slab surrounded by six outer wells at equal distance from the center. The six outer wells contained 5.0 µl of mouse monoclonal antibody isotyping reagent (Sigma) each specific to a particular type of immunoglobulin heavy chain. The resultant line of precipitation (Figure 1), demonstrated that the H12 monoclonal antibody (mAb) is a type IgG1 antibody.

Indirect Immunofluorescence Microscopy with H12 Hybridoma Supernatant

Coons pioneered the use of fluorescent derivatives of antibodies to trace antigen (Coons *et al.*, 1941, 1942; Coons and Kaplan, 1950; Coons, 1961). It was shown that antibodies could be coupled with β -anthracene or fluorescein isocyanate with the retention of antigen-binding properties, and that these antibodies could be used as sensitive probes to detect and localize antigen. Riggs *et al.* (1958) introduced the more stable and continent fluorescein isothiocyanate (FITC), which is the most popular fluorochrome used in fluorescent labeling experiments to date.

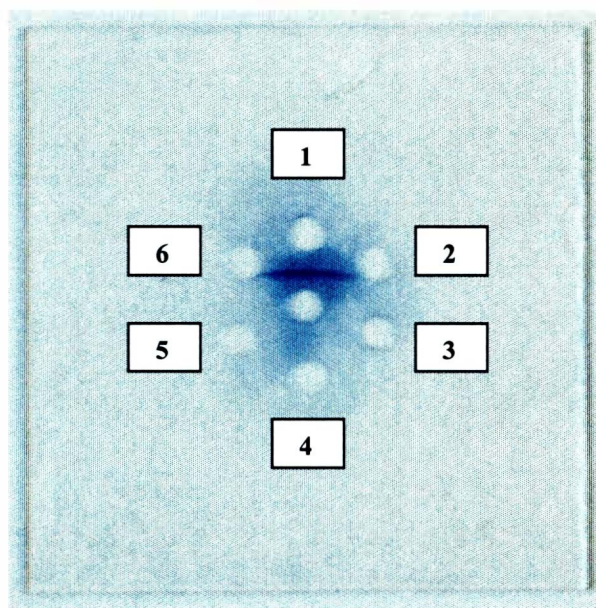


Figure 1. Ouchterlony Double Diffusion Analysis Demonstrating the Isotype of the Antibody Secreted by Hybridoma Clone H12. The wells contain the following isotype reagents (clockwise from top): 1) anti-mouse IgG1; 2) anti-mouse IgG2a; 3) anti-mouse IgG2b; 4) anti-mouse IgG3; 5) anti-mouse IgM and 6) anti-mouse IgA. The center well contains 25X supernatant collected from 75 m² tissue culture flask of hybridoma clone H12. The reagents were added, the agar incubated at 37° C for 24 hours and all non-conjugated protein eluted with PBS for 24 hour at RT. The agar then was dried, stained in 0.1% Coomassie Brilliant Blue in deionized water solution and destained in 5.0 % methanol, 7.0 % glacial acetic acid in deionized water solution.

An anti-mouse IgG (whole molecule) FITC conjugate (anti-mouse IgG-FITC) developed in goat (Sigma) was used to detect the binding pattern of the mAb secreted by the hybridoma clone H12 on YLDV-infected OMK cells and uninfected OMK cells by Immunofluorescence (Figure 2). At 7 days post-infection (dpi), Differential Interference Microscopy (DIC) of YLDV-infected OMK cells demonstrated CPE consistent with that observed for both TPV and YLDV in CV-1 cells (Knight *et al.*, 1989); a regressed monolayer with cytoplasmic granulation and vacuolization is clearly evident (Fig. 2. A and D). IF of YLDV infected OMK cells, at 7 dpi, treated with 25X H12 supernatant and anti-mouse IgG-FITC clearly demonstrate the areas of viral replication within the cytoplasm of the infected cells termed “viral factories” (Fenner *et al.*, 1989) (Fig. 2. B). An overlay of the DIC image and IF image for YLDV-infected OMK cells treated with 25X H12 supernatant and anti-mouse IgG-FITC demonstrates the enlarged viral factories within these cells clearly labeled with antibody, as evidenced by the areas of intense FITC staining coinciding with the areas of surface membrane protrusion (Fig. 2. C). IF of YLDV-infected OMK cells, at 7 dpi, treated with anti-mouse IgG-FITC only, fail to show binding to the viral factories (Fig. 2. E).

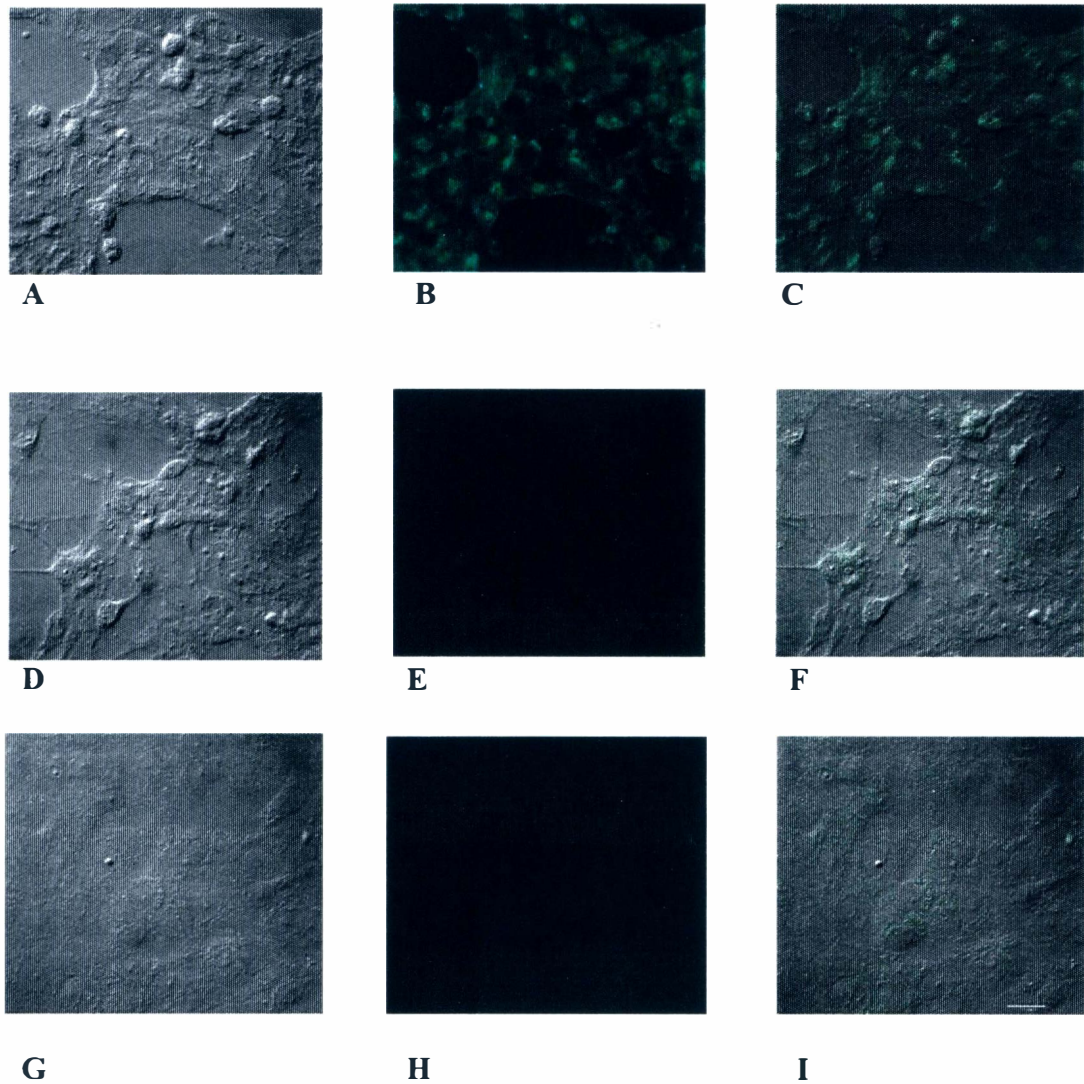


Figure 2. Indirect Immunofluorescence Microscopy Demonstrating the Selective Binding of H12 to YLDV Infected Owl Monkey Kidney Cells. Cell monolayers A, B, C, D, E, F were infected with YLDV at 50.0 pfu/cell and ethanol fixed at 7 dpi. Monolayers A, B and C were treated with 25X H12 supernatant and anti-mouse IgG-FITC. Monolayers D, E and F were treated with anti-mouse IgG-FITC only. Cell monolayers G, H and I are uninfected cells, treated with 25X H12 supernatant and anti-mouse IgG-FITC. Images A, D and G are DIC micrographs. Images B, E and H are IF micrographs. Images C, F and I are overlay micrographs of A-B, D-E and G-H respectively. Bar = 50 μ m.

In fact, IF images of YLDV infected cells treated with anti-mouse IgG-FITC only had staining patterns similar to that demonstrated for uninfected OMK cells treated with 25X H12 supernatant and anti-mouse IgG-FITC (Fig. 2. D, E, F, G, H and I). The results of the images obtained from IF demonstrate that the mAb secreted by hybridoma clone H12 selectively binds to YLDV-infected OMK cells and not uninfected OMK cells. Closer evaluation of these images also reveals that the areas of intense FITC staining are located only within the cytoplasm of YLDV-infected cells, while there is no evidence of staining within the nucleus of these cells. These combined results support the conclusion that the H12 mAb is directed against a YLDV antigen.

H12 Antibody is Directed Against a Late YLDV Polypeptide

Infection of mammalian cells with various members of the poxvirus group elicits the production of virus-induced proteins that differ in their time course of synthesis in infected cells (Wilcox and Cohen, 1967; Moss, 1996). The proteins that appear early during infection include enzymes responsible for nucleic acid metabolism (Jungwirth and Joklik, 1965) and virion structural proteins (Holowczak and Joklik, 1967a; Wilcox and Cohen, 1967; Sarov and Joklik, 1972; Fenger and Rouhandeh, 1976; Esposito *et al*, 1977; Kilpatrick and Rouhandeh, 1981; Vafai and Rouhandeh, 1982; reviewed in Moss, 1996). The late proteins which are those

appearing after viral DNA synthesis are incorporated into newly synthesized virus as structural components (Wilcox and Cohen, 1967).

Holowczak and Joklik (1967b) determined the temporal sequence of synthesis of vaccinia virus structural proteins by employing an inhibitor of DNA synthesis- cytosine arabinoside (Ara-C). We used this methodology to determine whether the target antigen of the H12 mAb was an early or late protein of YLDV. IF microscopy of YLDV-infected OMK cells treated with Ara-C at 0.0 hours post infection and YLDV-infected OMK cells not treated with Ara-C were ethanol fixed at 7 dpi and screened for antibody binding as previously discussed. The YLDV-infected OMK cells treated with Ara-C (Fig. 3. A, B and C) failed to demonstrate any sign of CPE even after 7dpi, as clearly evidenced in the DIC image of these monolayers (Fig. 3. A). In contrast, those cells that were infected with YLDV and not treated with Ara-C (Fig. 3. D, E and F) demonstrated classic CPE, as shown in the DIC image of these cells (Fig. 3. D). Fluorescent images of YLDV-infected OMK cells treated with Ara-C (Fig. 3. B) demonstrate no specific binding of the H12 mAb. While YLDV-infected OMK cells not treated with Ara-c (Fig. 3. E) show specific binding. The results of these experiments show that the H12 mAb is directed against a late protein synthesized following YLDV viral DNA synthesis. With that, this protein is most likely a structural protein of the YLDV virion (Wilcox and Cohen, 1967).

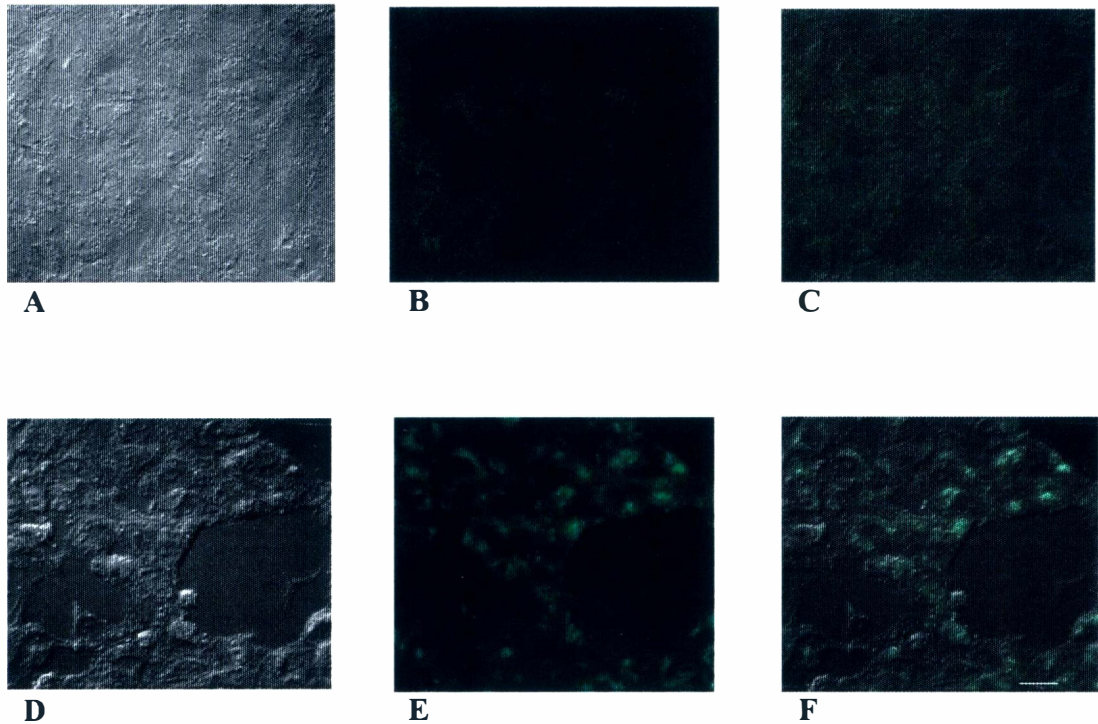


Figure 3. Indirect Immunofluorescence Microscopy Demonstrating H12 Binding Directed Against a Late YLDV Polypeptide. Cell monolayers A, B, C, D, E, and F were infected with YLDV at 50.0 pfu/cell and ethanol fixed at 7dpi. Images A, B and C represent cell monolayers that were treated with 40.0 µg/ml Ara-C at 0 hours post infection. Images D, E, and F represent monolayers that were not treated with Ara-C. A and D are DIC images, B and E are fluorescent images, and C and F are overlay images of A-B and D-E respectively. Bar = 50 µm.

H12 Antibody Demonstrates Cross Reactivity with TPV Through Indirect Immunofluorescence Microscopy

YLDV and TPV have previously been shown to share similar biological properties (Downie *et al.*, 1971; Espana, 1966; Hull, 1968; McNulty *et al.*, 1968), as well as being indistinguishable in serological experiments in an attempt to recognize different antigenic properties between YLDV and TPV (Downie and Espana, 1972).

IF microscopy was used to demonstrate the binding of the H12 mAb to TPV-infected OMK cells. TPV-infected OMK cell monolayers were ethanol fixed at 7 dpi, treated with 25X H12 supernatant and anti-mouse IgG-FITC. The results from these experiments are summarized in Figure 4. TPV-infected OMK cells treated with 25X H12 supernatant and anti-mouse IgG-FITC (Fig. 4. A, B and C) demonstrate identical binding patterns to that were seen with YLDV-infected OMK cells treated identically (Fig. 2. A, B and C). Also, TPV-infected OMK cells treated with anti-mouse IgG-FITC only (Fig. 4. D, E and F) show no specific binding, which is identical to the results from YLDV-infected cells treated similarly (Fig. 2. D, E, and F). These results demonstrate that the H12 mAb cross reacts with YLDV and TPV with resultant binding patterns that are indistinguishable. These results support those that have been previously reported for antigenic similarity between YLDV and TPV in complement-fixation tests, neutralization tests, precipitation tests and active immunity experiments (Downie and Espana, 1972).

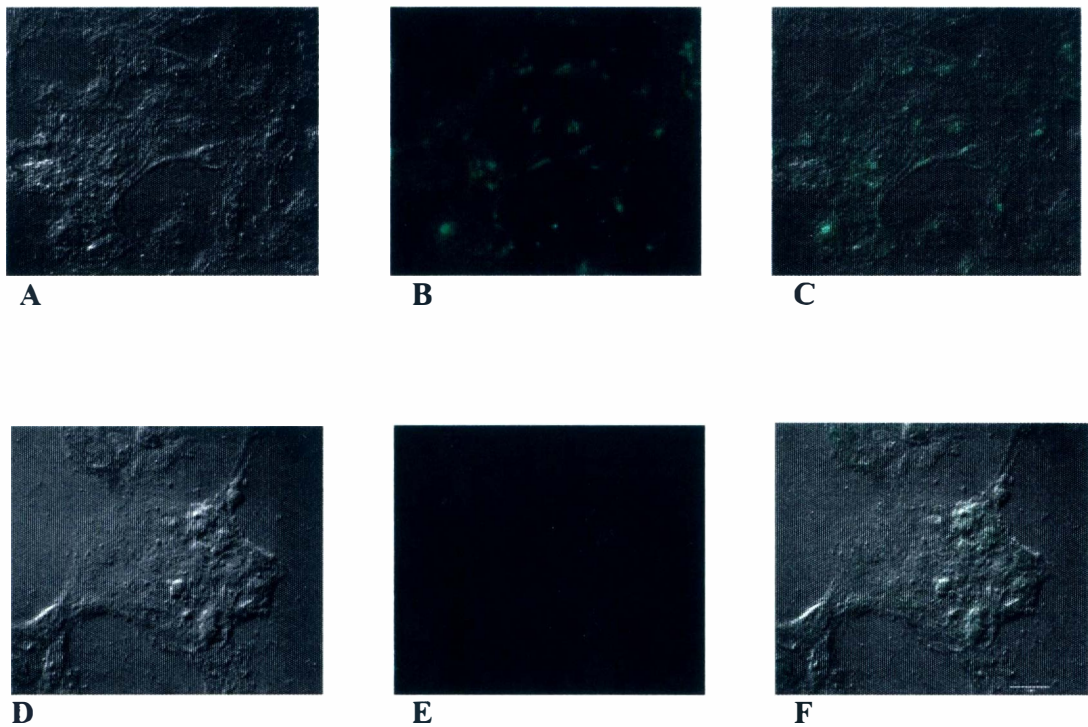


Figure 4. Indirect Immunofluorescence Microscopy Demonstrating H12 Cross Reactivity with TPV. Cell monolayers A, B, C, D, E, and F were infected with TPV and ethanol fixed at 7 dpi. Images A, B and C represent cell monolayers that were treated 25X H12 supernatant and anti-mouse IgG-FITC. Images D, E, and F represent monolayers that were treated with anti-mouse IgG-FITC only. A and D are DIC images, B and E are fluorescent images, and C and F are overlay images of A-B and D-E respectively. Bar = 50 μ m.

Immunoprecipitation of Viral Antigen with H12 Supernatant Demonstrates Two Polypeptide Chains Precipitated

Radiolabeling of the antigen provides a sensitive basis for its detection, and has the major attraction that non-labeled proteins such as antibodies may be added without detection in the final readout. This approach was pioneered by Schwartz and Nathenson (1971) and has been widely adopted to study hundreds of different antigens.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to resolve the viral antigen to which the H12 mAb is directed. Samples of soluble protein were prepared from YLDV-infected OMK cells and non-infected cells. The samples were then treated with either 25X H12 supernatant or a dilution of an anti-mouse IgG antibody (anti-mouse IgG) and allowed to precipitate over night. Following a series of centrifugation and wash cycles the samples, along with a high molecular weight standard, were then loaded on an 11.5 % polyacrylamide gel for resolution. The results from an SDS-PAGE of immunoprecipitates are summarized in Figure 5. The YLDV-infected cell sample treated with H12 supernatant (Fig. 5. lane 1) shows the presence of two distinct bands- a 40.7 kDa protein and a 26.9 kDa protein; that are not present in either the sample of YLDV-infected cells treated with the anti-mouse IgG (Fig. 5. lane 3) or the uninfected cells treated with H12 supernatant (Fig. 5. lane 4). Samples of the YLDV-infected cells and un-infected cells were centrifuged prior to the addition of H12 supernatant (Fig. 5. lanes 2 and 5 respectively) to remove all non-specific protein bands seen in previous experiments.

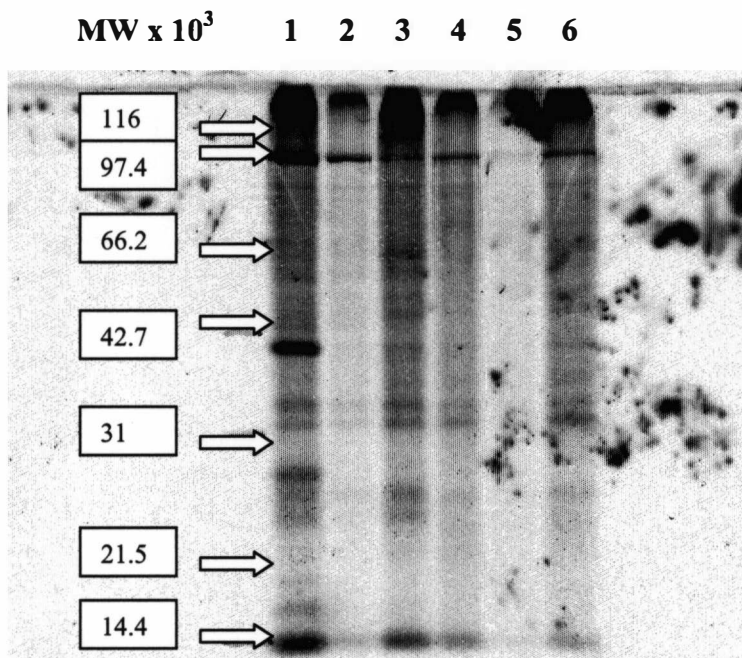


Figure 5.

Autoradiogram of Immunoprecipitates with H12 Demonstrating Precipitation of Two Polypeptide Chains. Cell monolayers infected with YLDV at 50.0 pfu/cell were radiolabeled with ^{35}S -methionine for 4.0 hours at 7 dpi (lanes 1, 2 and 3). Mock-infected cell monolayers were labeled with ^{35}S -methionine for 4.0 hours following 7 days in maintenance medium. Cells were harvested and plasma membranes lysed with 1.0 % NP-40 with 1.0:1.0 (v/v) Immunoprecipitation buffer added following treatment with detergent and removal of nuclei via centrifugation. An aliquot of 50.0 μl of YLDV-infected cells (lane 1) and mock-infected cells (lane 4) were treated with 50.0 μl of 25.0X H12 supernatant, 50.0 μl of YLDV-infected cell (lane 2) and mock-infected cells (lane 5) were centrifuged at 12,000 x g for 20.0 min prior to addition 50.0 μl 25.0X H12 supernatant, and YLDV-infected cells (lane 3) and mock-infected cells (lane 6) were treated with 50.0 μl of 1.0:20.0 dilution of $\alpha\text{-mIgG}$ in PBS; all samples were incubated at 37.0° C for 24 hours. An anti-mouse IgG in a 1.0:100.0 (v/v) dilution in PBS was added following 24 hours to all samples and incubated for an additional 4.0 hours at 37.0° C. Each sample was then centrifuged at 12,000 x g for 20.0 min and the pellet washed with 0.85 % (w/v) NaCl in deionized water 3X. The samples were then loaded on an 11.5 % polyacrylamide gel as described by Laemmli (1970). Standard molecular weights are indicated on the left in kilodaltons.

DISCUSSION

The results of the experiments performed for the completion of this project demonstrate the development of a single hybridoma clone H12, which secretes an anti-YLDV IgG1 isotype antibody that cross reacts with TPV. Difficulties experienced in the development of monoclonal antibodies against the YLDV virus may be due to the immunosuppressive properties of the virus itself. Throughout the course of immunization we did not observe any signs of viral replication in the immunized mice, so this suppression does not seem to be an active process in this model. With that, the immunosuppressive characteristics of viral antigens to evade detection of the host immune system seem to play the greatest role when considering the number of anti-YLDV hybridomas that were produced throughout seven separate fusions. The YLDV virion proved to be an extremely difficult particle to raise monoclonal antibodies against.

H12 has proven to be a stable cell line surviving cryopreservation and conserving its antibody secreting properties. Ouchterlony double diffusion assay has shown the antibody secreted by the H12 hybridoma to be an IgG1 isotype antibody. This antibody has been shown to specifically bind to areas consistent with those described for viral replication in YLDV and TPV infected OMK cells and not uninfected cells through Indirect Immunofluorescence microscopy. Ara-C inhibition of viral DNA synthesis was used to show that the viral antigen is a late protein in the

temporal sequence of YLDV replication. Also, an immunoprecipitation experiment has shown two polypeptides- a 40 kDa polypeptide and a 26.9 kDa polypeptide, to be resolved when YLDV infected cell plasma membranes and cytoplasmic extracts were precipitated with the H12 mAb. Although the results of the immunoprecipitation experiment presented seem clear, these results could not be repeated through numerous attempts. Modifications such as isotopes at various concentrations, separate immunoprecipitation buffers, various time intervals of starvation and labeling of the infected cells, differing concentrations and volumes of supernatant added to the sample, etc, were all attempted over the last year with out repeating the results presented with in this manuscript. It is not uncommon for monoclonal antibodies to fail to form precipitates *in vitro*, it has been previously demonstrated that seemingly minor modifications to antigen or antibody occurring for known for unknown reasons may result in complete abolishment of antibody binding (Nussenweig, *et al.*, 1982). A number of alternate experiments may resolve the viral antigen of the H12 mAb. A western blot of YLDV infected OMK cell and uninfected cell protein treated with the H12 mAb and the appropriate controls may lead to the confirmation of the target antigen. Another possibility would be to again attempt an immunoprecipitation experiment with purified H12 mAb via a protein A column. Also, to try to maximize the amount of antibody recovered, a buffer that contains high pH and high salt concentration may be a suitable choice given that mouse IgG1 antibodies have been shown to bind strongly in this type pf buffer solution (Goding, 1996). The purified antibody could then be titrated in order form optimum

precipitates and avoid the prozone phenomenon (failure to form precipitates due to antigen or antibody excess).

There are many potential uses for this antibody as a reagent in the basic research of both YLDV and TPV. The H12 mAb may possibly facilitate the elucidation of the molecular mechanisms of the virus-host interactions. It also demonstrates the potential for being used as a clinical reagent in the diagnosis of YLDV and TPV. Samples taken from those primates where infection from either of these two pathogens is suspected could be properly prepared and stained with the H12 mAb to detect the presence of either YLDV or TPV.

Appendix A
IACUC Approval Letter

WESTERN MICHIGAN UNIVERSITY
YEARLY RENEWAL FORM APPLICATION TO USE
VERTEBRATE ANIMALS FOR RESEARCH OR TEACHING

GENERAL INFORMATION: Fill in all appropriate information



<u>Karim Essani</u> Principal Investigator/Instructor	<u>Biological Sciences</u> Department	<u>7-2661</u> Campus Phone
<u>Scott Haller (Student)</u> Co-Principal/Student Investigator	<u>Biological Sciences</u> Department	<u>7-2661</u> Campus Phone

Title of Project/Course Preparation of Monoclonal Antibodies

PRINCIPAL INVESTIGATOR/INSTRUCTOR DECLARATION

I assure that I have obtained IACUC approval prior to implementing this project and that there are no changes in the protocol submitted in the original application to use vertebrate animals for research or teaching. I understand that if at any time changes are made in the use of animals as described in the original application, a letter or amended protocol must be filed for review. I assure that the activities do not unnecessarily duplicate previous experiments.

Signatures:

<u>Karim Essani</u>  Principal Investigator/Instructor	Feb. 29, 2001 Date
<u>Scott Haller</u>  Co-Principal/Student Investigator (If PI not a faculty member)	Feb. 29, 2001 Date

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE APPROVAL

 IACUC Chairperson	<u>2/27/01</u> Date
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PLEASE MAIL COMPLETED APPLICATION TO:

Research Compliance Coordinator
Western Michigan University
327E Walwood Hall
Kalamazoo, MI 49008
(616) 387-8293

IAC-F

ALL THREE DATES ARE ACTUALLY MARCH 1, 2001 JES

Appendix B
Radiation Safety

A WORLD OF DIFFERENCE



WESTERN MICHIGAN UNIVERSITY

CERTIFICATE FOR AUTHORIZED USER OF RADIOACTIVE MATERIALS

This certifies that SCOTT HALLER has completed a 4-0 hour radiation safety training course for sealed and unsealed radioactive materials.

I am hereby designated and responsible for the radiation safety training information that I have received as it pertains to the ALARA (as low as reasonably achievable) radiation exposure practices for supervision and/or use of radioactive materials and/or radiation machines. I agree to follow the rules and regulations of the Western Michigan University Radiation Safety Program, State of Michigan's "Ionizing Radiation Rules," Michigan's Department of Consumer & Industry Service and Department of Environmental Quality rules and regulations, and the U.S. Nuclear Regulatory Commission's licensing specifications. Further, I have read and understand the information that has been given to me at the Radiation Safety Training course for Sealed and Unsealed Radioactive Materials.

This certificate is issued as it specifically pertains to the use of: UNSEALED RADIOACTIVE MATERIALS

This certificate expires on: 9/9/98

I agree to attend a refresher radiation safety training course prior to the date of expiration in order to continue to use radiation machines or radioactive materials at W.M.U.

Date of Birth: 0000 Social Security #: 000000

<u>SCOTT HALLER</u>	<u>9/9/97</u>
Name of Recipient	Date
<u>[Signature]</u>	<u>9/16/97</u>
Signature of Instructor	Date
<u>[Signature]</u>	<u>9/9/97</u>
Phil Noack, R.S.O.	Date

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