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Development and Characterization of Monoclonal Antibodies Directed Against Tanapox Virus

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DEVELOPMENT AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES DIRECTED AGAINST TANAPOX VIRUS

by

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The initial symptoms of a Tanapoxvirus (TPV) infection are indistinguishable from smallpox, which is caused by variola virus (VV). In the post-9/11 area, reagents that can quickly differentiate a TPV infection from smallpox are very desirable, but currently unavailable. Monoclonal antibodies (mAbs) directed specifically against TPV can be used for this purpose. Six mAbs were developed and characterized that specifically bind to TPV infected owl monkey kidney (OMK) cells, but not uninfected cells. The apparent molecular mass of the TPV protein(s) recognized by 5 mAbs were calculated using the Western blot technique. One mAb demonstrated no binding using the Western blot technique, but demonstrated specific binding to TPV protein(s) by ELISA and indirect immunofluorescence microscopy. Since TPV is included in the genus *Yatapoxvirus* and the VV is included in the genus *Orthopoxvirus*, with no immunological cross-reactivity, it is presumed that these antibodies will react with TPV proteins and not VV proteins.

TABLE OF CONTENTS

Table of Contents-Continued

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Tanapoxvirus (TPV) is a large DNA containing virus included in the family *Poxviridae,* where it has been classified as a yatapoxvirus. TPV causes a mild, self-limiting, febrile infection in humans with very few skin lesions (Downie *et al.*, 1971). Morphologically TPV is very similar to variola virus (VV), the causative agent of smallpox, and cannot be differentiated from other poxviruses based on electron microscopy. Smallpox is the most feared disease of the post-9/11 era. Although TPV infection is endemic to Africa, recently there was one case in Europe (Stich *et al.*, 2002) and another in the United States (Dhar *et al.,* 2004) where individuals returning from Africa showed classical poxvirus symptoms and the virus appeared to be similar in morphology to VV. It was, therefore, necessary to have essential diagnostic reagents at hand to rule out smallpox. The TPV genomic sequence data generated in our laboratory (Neering, 1993; Paulose *et al.*, 1998) were very useful in confirming a TPV infection and ruling out smallpox.

The purpose of this study was to generate even simpler reagents, monoclonal antibodies (mAbs), to facilitate simpler, faster and more cost-effective methods to differentiate a TPV infection from a VV infection. Here I describe the generation and characterization of six anti-TPV monoclonal

antibodies that can be potentially used for this purpose. These antibodies specifically recognize TPV antigens. Since VV (an orthropoxvirus) and TPV (a yatapoxvirus) are antigenically distinct based on immunological cross reactivity. We believe these mAbs will be very useful to differentiate TPV from VV. Unfortunately federal law prohibits us from working with VV to provide evidence for this notion. Perhaps in the near future the Centers for Disease Control and Prevention (CDC) in Atlanta, one of the two laboratories in the world with VV, will be able to confirm our findings.

REVIEW OF LITERATURE

Poxviruses

Poxviruses are among the most complex and perhaps the most studied of all known animal viruses. A member of the family *Poxviridae*, VV is the causative agent of the disease smallpox the most feared disease of all time. Smallpox was declared eradicated from the face of the globe in1977 as a result of a rigorous vaccination program and is considered one of the major achievements of modern science. Fears of bioterrorism in the post 9/11 era has, however, raised speculation about the intentional release and reemergence of smallpox in the global population without any specific immunity to smallpox

Basic research on vaccinia virus (VAC) has greatly pioneered our understanding of viruses. The eradication of smallpox was achieved through injections of the closely related vaccinia virus, a poxvirus antigenically similar to VV, coining the term vaccination. The process of vaccination to prevent infectious diseases is common practice today, but it was initially pioneered, over 200 years ago, by Edward Jenner after his observation that milkmaids who had exposure to cowpox seemed to have immunity against smallpox. Vaccinia virus was also the first animal virus seen microscopically and

extensively studied. The discovery that purified VAC contains enzymes that synthesize mRNA (Kates and Beeson, 1970; Golini and Kates, 1985) changed the overly simplistic view that viruses are just little bundles of nucleic acids. Research on poxviruses has spearheaded our understanding of viral pathogenesis. The discovery and study of viral encoded proteins may provide new insights into virus/host relationships as well as elucidate the mechanisms of the host immune system.

All members of *Poxviridae* are large complex virions containing a single linear double stranded DNA molecule that may range in size from 130 kbp to 300 kbp, as well as enzymes that synthesize mRNA. Poxviruses go through two distinct stages of uncoating. The first uncoating process occurs immediately upon entry into the cytoplasm of the host cell. The second stage requires proteins produced by the early transcription system contained within the virus to take place. The enzymes contained within the virus are responsible for the synthesis of viral mRNA and the processing of that viral mRNA in a manner that is similar to eukaryotic mRNA. The processed viral mRNA is capped and methylated (Wei and Moss, 1975). The viral mRNA also has a poly A tail (Kates and Beeson, 1970). Vacinia virus early mRNA can be detected in the cytoplasm within 20 minutes of infection and accumulates to its highest levels within 1-2 hours. The amount of viral mRNA declines in concentration at a similar rate with measurable amounts present at 4 hours post infection (Baldick *et al.*, 1992). The viral mRNA produced

before the second stage of uncoating are classified as early viral mRNA and the proteins encoded by this early viral mRNA are classified as early viral proteins. These early proteins include growth factors and immune defense molecules as well as proteins required for the second stage of uncoating and later DNA replication (reviewed in Moss, 1990 and Traktman, 1990). After the second stage of uncoating, the initiation of viral DNA replication can take place (Broyle, 1993). The viral DNA is replicated in the cytoplasm in localized areas called factories, which become larger and more pronounced as the infection continues. The cytoplasmic sites of replication, as opposed to within the nucleus, is a characteristic trait of all poxviruses and a trait that makes them unique among viruses. The cytoplasm of the host cell is also the site of viral assembly. Two distinct forms of infectious particles can be seen, intracellular mature virions (IMV) and extracellular enveloped virions (EEV) which travel to the cell surface using microfilaments (Hiller, *et al.*, 1981) and leave the host cell by budding out. The EEV have an additional lipoprotein envelope, derived from the host cell membrane, when compared to IMV (Payne, 1978). There are differences in the pathogenesis of IMV and EEV and differences in the surface proteins of each, but both types of particles can cause infection.

Immediately following infection and for a variable amount of time afterwards the infected host cell cannot be differentiated from a non-infected host cell on the basis of any observable characteristic. As a viral infection

progresses, the changes seen in the host cell are termed the cytopathic effect (CPE). The characteristics of the CPE that is observed during infection is a function of the type of virion and the type of host cell infected. Poxvirus infection causes the host cell to change its cytoskeletal organization and overall morphology as the infection progresses (Traktman, 1991). The morphological changes observed include granulation of the cytoplasm due to the increasing viral factories and accumulating viral particles as well as the rounding and retraction of infected cells followed by possible detachment of infected host cells from the surface of the flask during later stages.

Yatapoxviruses

Yaba monkey virus (YMTV), tanapoxvirus (TPV) and yaba-like disease virus (YLDV) are the members of the genus *Yatapoxvirus* belonging to the family *Poxviridae*. *Yatapoxvirus* genus viruses infect all primates, but are thought to mainly infect non-human primates. YMTV was first isolated from tumors that occurred in a colony of monkeys is Nigeria (Niven *et al.*, 1961). No evidence of human infection with YMTV in nature has been reported, but subcutaneous injection produces tumors in the skin of monkeys and human beings (Fenner, 1996). TPV and YMTV show minimal to moderate serological cross reactivity. Primates first infected with TPV and later challenged by YMTV, showed delayed and diminished symptoms, while TPV

and YLDV show more significant cross reactivity with TPV infection fully protecting primates against YLDV challenge (Downie and Espana, 1973). A mAb generated against YLDV shows cross reactivity with TPV (Haller, 2001). Some authors (Fenner, 1996) have referred to TPV and YLDV to be the same virus, but there is data to suggest that TPV and YLDV are separate, but closely related strains, both distinctly different from YMTV. The DNA restriction maps of TPV and YLDV show only minor differences between them while YMTV showed more significant differences when compared to both TPV and YLDV (Knight *et al.*, 1989).

Tanapoxvirus and Yaba-like Disease Virus

TPV was first identified in a village on the flood plains of the Tana River valley in Kenya. TPV was originally isolated from human skin biopsy specimens taken during a much more widespread outbreak of disease among residents of the Tana River Valley 1962 (Downie *et al.*, 1971). This epidemic was associated with flooding in Kenya when the human population, domestic animals and the wild animal population were crowded together on small islands among the floodwaters. Subsequent serological studies showed that it was endemic to this area and many subsequent cases were identified during surveillance for monkeypox in Zaire from 1977 to 1981. The majority of the cases identified in Zaire occurred among people living in close

proximity to the Zaire River and more frequently in people who worked close to the river as opposed to working in the upland agricultural fields (Jezek *et al.*, 1985). An arthropod vector, possibly mosquitoes, is thought to be the culprit responsible for the transmission of TPV to humans from its reservoir among the wild animal populations.

In 1965 and 1966, animal handlers at primate centers in California, Oregon and Texas contracted a poxvirus disease through cuts or abrasions that happened when handling infected monkeys or cleaning cages that housed infected monkeys (Crandell *et al.*, 1969). The virus isolated from these monkeys was initially shown to be indistinguishable in its serological properties to TPV isolated in 1962 from the infected natives in Kenya (Downie and Espana, 1971). Isolates from these cases have been described as "Yaba-like disease virus" and "Yaba-related virus" (Crandell *et al.*, 1969) because the lesions formed appear similar to those seen in YMTV. These isolates have also been called "Oregon '1211' poxvirus" (Nicholas and McNulty, 1968) and "OrTeCapox virus" since the same strain appeared to have been spread to Oregon, Texas and California carried by monkeys from the same animal supplier. Because the virus isolated from monkeys in American primate centers is antigentically very similar to the TPV isolated from humans, they have been considered a single species (Downie and Espana, 1973, Fenner, 1996). Further research has shown that the TPV isolated from humans in Kenya and the virus isolated from monkeys at the

American primate centers to be very closely related, but not identical (Knight *et al.*, 1989, Lee *et al.*, 2001). For clarity, this paper will refer to isolates from humans in the Tana River valley, Kenya as TPV and isolates from monkeys and animal handlers at the American primate centers in 1965-1966 as Yaba-like disease virus, or YLDV.

The last known case of TPV within the United States was from a student in New Hampshire who had recently returned to the United States from Africa in the fall of 2002 (Dhar, *et al.*,). She had spent eight weeks serving and caring for orphaned chimpanzees in the Republic of Congo. Initially, health care professionals were greatly concerned about a potential smallpox outbreak given the symptoms of the patient and the current concerns of the potential use of VV as a weapon of bioterrorism. Electron micrographs of TPV and VV appear identical, but the CDC later correctly identified that case as a TPV infection and a clinical isolate from that individual currently resides in the laboratory of Dr. Karim Essani.

TPV replicates in a manner similar to other poxviruses, but the replication cycle is longer when compared to VV (Mediratta and Essani, 1999). TPV does cause disease in humans, but in contrast to smallpox, the infection is usually self-limiting in people with competent immune systems and a pustule is only formed at the site of infection. The initial skin lesion is a small raised nodule that appears to be similar to what is normally seen with an insect bite and gradually enlarges for about two weeks. The disease is

characterized by fever, severe headache, backache and flu-like symptoms lasting less than a week. The lymph nodes draining the initial site of infection are enlarged and tender from about the fifth day after the appearance of the skin lesion which usually ulcerates during the third week and then heals within 5 to 6 weeks leaving a scar.

Part of the genomic sequence of TPV is known (Neering, 1993). The genomic sequences for YMTV, YLDV, VV and many other pox viruses have also been published and are readily available enabling the study of the homology among poxvirus encoded proteins (Lee *et al.*, 2001, Brunetti *et al.*, 2003, Gubser *et al.*, 2004). TPV, like some other poxviruses, has been shown to secrete immunomodulatory proteins that inhibit host immune responses (Essani *et al.*, 1994, Paulose *et al.*, 1998, Smith *et al.*, 1998, Smith *et al.*, 2000, Brunetti *et al.*, 2003). Research into the structure and function of these proteins provide an excellent opportunity to provide new information as to the intricacies of the immune system. The relatively low risk of handling cultures and the published genomic sequences make TPV a good model for studying a member of the *Poxviridae*, the proteins that are encoded by its genome and their role in the virus-host relationship.

Antibodies

History of Antibodies

Since ancient times it has been known that previous exposure to certain diseases gives the survivors protection against contracting those diseases later in life. An infant or young child is susceptible to many infections, but with the aid of a functioning immune system, the child will develop specific immunity to many of the pathogens once their body has successfully dealt with them. The immune system has memory. The survivors are still prone to contracting many other diseases, which demonstrates another key concept of the immune system. The immune system is specific, or in other words, there is immunological specificity.

The blood has long been the focus of the study of immunity. In 1794, John Hunter observed that blood did not putrefy as rapidly as other tissues. In 1888, Nuttall observed that the serum from normal, healthy animals could sometimes kill micro-organisms. In 1890, von Behring and Kitasato demonstrated that immunity to diphtheria and tetanus was attributed to antibodies to the toxins that are produced by each of those pathogens (reviewed in Goding, 1996). The medical application of these findings resulted in a wide range of outcomes. Using the serum from an immunized

animal to treat a human or animal that had not been immunized, called "serum therapy", sometimes lead to dramatic recovery. In other cases it resulted in a syndrome known as "serum sickness" with skin rashes and arthritis the result of the development of massive amounts of antigen-antibody complexes. Occasionally sudden death due to what we now call anaphylactic shock was the unfortunate result. Paul Ehrlic showed that there was a quantitative relationship between the plant toxin ricin or abricin and the amount of antibody needed to neutralize their toxicity. To Ehrlich, this suggested a chemical reaction between antigen and antibody. Ehrlich also championed the notion that these chemical reactions involved specific binding and proposed the "lock and key" analogy when referring to antigen-antibody interactions. With the scientific advancements in the area of genetic theory, protein structure and the reconciliation of the many observations of cellular and humoral immunity, antibodies and the B-lymphocytes that produce them became a large focus of study on the specificity and memory aspects of the immune system.

Monoclonal Antibodies

It is now well demonstrated that the sera of an animal is polyclonal with each B-lymphocyte predetermined to make one and only one antibody that recognizes a single specific complimentary antigen and at each particular

time each clone population is some percentage of the total B-lymphocyte population. According to the clonal expansion theory, when an animal is immunized with an antigen, the clonal expansion of those B-lymphocytes that recognize the antigen and their differentiation into antibody secreting cells takes place. If individual antigen recognizing B-lymphocytes could be separated and propagated continuously *in vitro* the culture supernatant would contain homogeneous antibody molecules that recognize one or a few very closely related antigens. Unfortunately the progeny of an untransformed lymphocyte cannot be grown continuously *in vitro*. The progeny of transformed, or immortal, B-lymphocytes can be grown continuously *in vitro* producing large amounts of monoclonal antibody (mAb). In 1975 the first immortalized antibody secreting lymphocytes were created by fusing immortalized lymphocytes with untransformed antibody secreting lymphocytes (Kohler and Milstein, 1975). These hybrid cells whose parents were an immortal myeloma cell and secreting B-lymphocyte are called hybridomas. These hybridomas can then be grown from individual cells producing monoclonal cell lines that secrete one particular antibody molecule.

When two antibody producing cells are fused, the resulting hybridoma cell expresses the antibodies from both parental lines, although the light and heavy chains of both parental lines are randomly joined, showing no evidence of scrambling of the variable or constant regions are observed. In other words, even though each antibody may be a mixture of polypeptides with

some coded for by the genes for one parental line and some coded for by the other parental line, each individual polypeptide chain was entirely coded for by a single parental line (Cotton and Milstein, 1973). Milstein had been interested in the genetic control of antibody synthesis. Cotton and Milstein (1973) constructed hybrids between rat and mouse myeloma cells and found that the synthesis of both species of antibody was retained, but hybrid polypeptides that would have some amino acids coded for by rat DNA and some coded for by mouse DNA were not seen. These results were consistent with the idea that the variable region and constant region joining took place at the DNA level and not on the mRNA level. For the production of mAb that is identical to the secretory lymphocyte, an immortalized myeloma cell that is non-secreting would make an ideal fusion partner with the resulting hybridoma secreting only the mAb from the lymphocyte parental line. A myeloma cell line, P3-X63-Ag8.653, which does not express heavy or light chains was discovered in 1979 and was shown to be an ideal fusion partner (Kearney *et al.*, 1979).

Cell Fusion and Hybridoma Selection

Sendai virus was the original agent used to fuse cells to create hybridomas. Electroporation (Steenbakkers *et al.*, 1993) and electroacoustic (Bardsley *et al.*, 1990) are relatively newer techniques that may be useful

when low numbers of B-lymphocytes are available for fusion. The most traditional method of fusion involves using polyethylene glycol (PEG) to fuse the membranes of two cells together. The exact mechanism of fusion is still poorly understood, but presumably PEG promotes close apposition of adjacent cell membranes (Zimmerberg et al., 1993).

If a mixture of cells containing myeloma cells and secretory B cells is treated with agents that promote fusion, the resulting fusion products are random and poorly controlled. In addition to the myeloma-B cell hybrids that are the desired goal, there will be many myeloma-myeloma and B cell-B cell fusion products in addition to fusion products that result from the fusion of several cells. Individual myeloma and B cells that did not undergo any fusion will also be present. In order to produce a long term hybridoma cell line that secretes mAb, a selection process is required. The most common selection involves the blocking of nucleotide biosynthetic pathways by the careful selection of fusion partners and by use of a selective medium. This process was originally devised by Littlefield (1964). This selection procedure is based on the fact that when the main biosynthetic pathway for guanosine synthesis is blocked by the folic acid antagonist aminopterin. There is an alternate "salvage" pathway in which the nucleotide metabolite hypoxanthine is converted by the enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT) to guanosine monophosphate providing the essential nucleotide despite having the main pathway blocked. Cells lacking a functional HPRT

enzyme die in a medium containing hypoxanthine, aminopterin and thymidine (HAT medium). HAT medium selects for HPRT(+) cells because aminopterin blocks the main pathway. The parental B cells will be HPRT(+) and all combinations of fused B cells as well as non-fused B cells will not be killed by the selective HAT medium, but because they are not transformed cells they will only survive a few weeks at most *in vitro*. All combinations involving HPRT(-) myeloma cells, fused or un-fused, will be all be killed by HAT medium. HPRT(-) meyloma cells can be selected for prior to the fusion by the use of the toxic base analogs 8-azaguanine or 6-thioguanine which are incorporated into DNA by HPRT. Because the salvage pathway is not normally essential for survival, mutants that lack HPRT +will continue growing while HPRT(+) cells will die. Only the fusion products involving the immortal myeloma cells and the HPRT(+) B cells will survive indefinitely in HAT medium. The resulting HPRT+ hybridomas can be cloned by limited dilution to create uniform cultures that produce mAb. Selection for HPRT(-) myeloma cells prior to fusion is essential due to the fact that myeloma cells will spontaneously mutate back to HPRT(+) cells (Littlefield 1964). Enough time must be allowed to clear the medium that the HPRT(-) myeloma cells are grown in of 8-azaguanine because it is toxic to the HPRT(+) hybridomas which will be produced later.

Impact of Monoclonal Antibodies

Monoclonal antibodies (mAbs) have had revolutionary consequences in biological research and clinical medicine and has resulted in an industry that has an economic impact measured in billions of dollars (Chien and Silverstein, 1993). The specific binding properties of mAbs make them a useful research tool for the identification and analysis of proteins (Singh *et al.* 2003 and Tanimura *et al.*, 2004). The genetic revolution has had an extremely profound effect on biological research and its applications, but all the information contained in any gene sequence is in most cases only relevant once it is expressed as protein. DNA has proved to be the blueprint of life, but proteins are still the structure and functional machines of life. One reason the notion that nucleic acids were the molecules conveying heredity was so elusive was because so much focus was placed on proteins. Proteins are literally found everywhere and do everything in biological systems. Even though the amino acid sequence of a multitude of genes are literally a mouse click and a BLAST search away, this information is greatly complicated by post-translational modifications, protein folding and three dimensional structure. A gene sequence is not the end of biological research on the function and effects of that gene, it is often just the beginning. The specificity of mAb technology provides a means to identify and/or purify a single protein

out of a complex mixture of molecules. They can be used to identify and study novel proteins of micro-organism or macro-organism origin, greatly aiding the advancement of basic scientific research which is the backbone of literally all technological advances. The use of mAbs in the generation of enzyme linked immunosorbent assays (ELISAs) has dramatically improved the quality and efficiency of many standardized tests and clinical diagnostic assays. mAbs conjugated with a radioactive, or fluorescent label can be used to not only detect the presence of a protein, but the location as well. The indirect immunofluorescence images presented later in this manuscript were made using this technique. Using the specificity of mAbs to target cancer cells has had its successes as well as failures, but continues to be an area of intense research. The net effects resulting from the generation of a novel mAb is unknown, but it would no doubt be a welcome addition to the legacy of mAb technology.

MATERIALS AND METHODS

Virus, Cells, Reagents and Animals

Owl monkey kidney (OMK) cells obtained from the American Type Culture Collection (ATCC, Rockville MD) were grown in the growth medium defined as: EMEM with 10% (v/v) newborn calf serum (NBCS), 0.2 mM L-glutamine and antibiotics (100units/ml penicillin G sodium, 100 μg/ml streptomycin sulphate and 0.25 μg/ml amphotericin B). Infected and mock infected OMK cells were maintained in the maintenance medium that contained 2% (v/v) NBCS instead of the 10% in the growth medium. TPV was cultivated in OMK monolayers as described later. Mouse myeloma cells, P3x63.Ag8.653 (ATCC) cells were grown in RPMI 1640 supplemented with 10%(v/v) FBS, 0.2 mM L-glutamine and antibiotics. All myeloma, hybridoma and OMK cells were incubated at 37°C and 5% CO_2. All tissue culture media, sera and reagents were purchased through Sigma (St. Louis, MO). Six BALB/c female mice, 4-6 six week old (Charles River laboratories, Portage MI) were kept at the animal facility in Haenicke Hall at Western Michigan University. All animals were monitored and treated in accordance with the approved IUCUC protocol number 03-01-01.

Virus Cultivation

Confluent monolayers of OMK were infected with either purified TPV (ATCC), or a mixture of TPV and cellular debris from previously TPV infected OMK cells. After adsorption at room temperature (unless otherwise stated) for 90 minutes. The infected preparations were incubated in maintenance medium at 37° C and 5% CO_2 for 10-14 days. Infected cells from monolayers showing advanced CPE were harvested using a sterile cell scraper and collected as a 1X virus preparation.

Virus Quantification

Quantification of viral preparations were performed by viral plaque assay (Essani, 1982). Confluent OMK monolayers grown in 6-well plates were infected with 200.0 µl per well of serial ten-fold dilutions of the viral prep. Each dilution was plated in duplicate. The series of 6-well plates were placed on a rocker table for virus adsorption at room temperature for 90 minutes. Following adsorption, 3.0 ml of maintenance medium with 0.5% methyl cellulose (4000 centipoises) (overlay medium) was carefully added to each well. The infected preparations were incubated at 37° C and 5% CO_2 for 10-14 days. The overlay medium was then aspirated from each well and 1.0 ml

of 0.1% crystal violet and 10% formaldehyde in deionized water was then added to each well to fix and stain the cells. The stain was removed and the plates were rinsed with distilled water and allowed to dry. The number of plaques in each well were counted. The average number of plaques for each dilution were calculated and multiplied by the dilution factor to calculate the plaque forming units (pfu) per ml of the original preparation.

Preparation of Monoclonal Antibodies

Preparation of Innoculum and Immunization of Mice

Four confluent monolayers of OMK cells (3×10^5) in 35 mm dishes were infected with TPV (10 pfu/cell). Once all of the OMK cells showed uniform CPE (4-5 days), the infected cell monolayers were washed three times with phosphate buffered saline (PBS). Sterile deionized water (200.0 μl) was added to each dish and the attached cells were scraped with a cell scraper to suspend the cells. The contents of each dish was collected and pooled and sterile dionized water was added to a total volume of 1.5 ml and stored at

-20°C. Six BALB/c female mice were immunized by a series of five weekly intraperitoneal injections of 100.0 μl of the prepared innoculum as described in Current Protocols in Immunology (Coligan, *et al.*, 1996). No adjuvants of

any kind were used. A final booster injection was given three days prior to fusion. All animals were monitored and treated accordance with the approved IUCUC protocol number 03-01-01.

Preparation of Myeloma Cells and Feeder Layers

Two weeks prior to fusion the myeloma cells were grown in medium supplemented with $6.6x10^{-2}$ M 8-Azaguaninine (Sigma) to kill any HPRT(+) revertants. After 2-3 cycles in medium containing 8-Azaguaninine, the myeloma cells were grown in normal medium without 8-Azaguaninine for 2-3 cycles. The cells were propagated in fresh growth medium one day prior to the fusion to ensure that the cells were in the exponential growth phase. The live myeloma cells were counted using a hemocytometer and trypan blue to ensure a 1:1 ratio of immunized splenocytes to myeloma cells (Freshney, 1994). Each mouse spleen was estimated to have 1×10^8 splenocytes (Coligan *et al.*, 1996). Twenty four hours prior to fusion, "feeder cells" were obtained from a non-immunized female BALB/c mouse. The mouse was sacrificed by cervical dislocation and 1.0 ml of PBS was injected intraperitoneally and then the abdomen was massaged. After rinsing with 70% ethanol, the peritoneal cavity was opened with sterilized scissors. A sterile, cotton plugged, glass pipette was used to remove the PBS containing the feeder cells and added to a centrifuge tube with 50.0 ml of hybridoma

growth medium. The peritoneal cavity was washed with this solution to remove as many cells as possible. Care was taken to avoid scraping the walls of the cavity in an attempt to minimize the number of fibroblasts collected. 100.0 μl of this feeder cell preparation was distributed to each well of five flat-bottom tissue culture plates using a multichannel pipette. The feeder cell plates were incubated overnight at 37°C and 5% CO_2.

Fusion

An immunized female BALB/c mouse was sacrificed by cervical dislocation and rinsed with 70% ethanol. The spleen was removed and transferred to a 50.0 ml centrifuge tube containing 5.0 ml of 37°C serum free medium (supplemented with antibiotics only). The spleen was then placed on a sterile, stainless steel mesh on top of a glass beaker in a laminar flow hood. A rubber policeman was used to homogenize the spleen. The spleen cells were washed with additional serum free medium. The contents of a prepared flask of myeloma cells was added to this mixture and centrifuged (75xg Damon IEC HN-SII, 1500 rpm) for 10 minutes. The supernatant was removed and the pellet was gently resuspended in serum free medium. The splenocyte/myeloma cell mixture was washed in this manner two more times in an attempt to remove FBS proteins from this mixture. Following the third wash, the supernatant was removed leaving approximately 100.0 μl of serum

free medium to resuspend the pellet. A beaker containing 37°C water was placed in the laminar flow hood and the tube was placed in it to stabilize the temperature. 1.0 ml of polyethlyene glycol (PEG) was added to the resuspended cells over the course of 1 minute. A controlled 1:50 dilution of the PEG and suspended cell mixture with serum free medium was carried out as follows: 10.0 ml over 10 minutes; 10.0 ml over 5 minutes; 10.0 ml over 3 minutes; 10.0 ml over 2 minutes; 9.0 ml over the last 1 minute. This mixture was then centrifuged (75xg Damon IEC HN-SII, 1500 rpm) at room temperature for 10 minutes. The supernatant was discarded and the pellet resuspended in 50.0 ml of hybridoma growth medium. 100.0 μl of this fusion preparation was added to each well of the 96-well feeder plates using large bore pipette tips (BIO-RAD, Richmond, CA, catalog number 223-9312) to reduce the physical stress on the freshly fused cells. These plates were incubated overnight at 37°C and 5% CO_2. Following incubation, 100.0 μl of hybridoma growth medium supplemented with $2x10^{-4}$ M hypoxanthine, $8x10^{-7}$ M aminopterin and $3.2x10^{-5}$ M thymidine (HAT) (2X selection medium) was added to each well to select for HPRT(+) cells. Three days after the addition of HAT medium, 100.0 μl of supernatant was removed from each well and replaced with 1X HAT medium to decrease the amount of dead cell byproducts in each well. After seven days all media changes were done with hybridoma growth medium supplemented with 2×10^{-4} M hypoxanthine and $3.2x10^{-5}$ M thymidine (HT) only.

Initial Screening

Confluent OMK monolayers were grown in 96 well plates and half of the plates were infected as described earlier. Infected and uninfected plates were kept in maintenance medium until 95% of the cells demonstrated CPE. The maintenance medium was removed and 100% ethanol at -80°C was added to each plate for 30 seconds to fix the cells. The ethanol was aspirated and the cells were washed with PBS with 0.1% gelatin (BIO-RAD, Richmond, CA) to block any non-specific protein binding. 100.0 µl of 1X hybridoma supernatant from wells to be tested was added to wells in both the infected and uninfected plates and incubated at room temperature for 4 hours. The supernatant was aspirated and the wells were washed three times with PBS with 0.1% gelatin. The secondary antibody, anti-mouse IgG alkaline phosphatase conjugate developed in goat (Sigma) was diluted 3:5000 in PBS with 0.1% gelatin and 50.0 µl was added to each well and incubated at room temperature for 4 hours. Each well was washed with PBS with 0.01% tween 20 to remove any unbound secondary antibody. 100.0 µl of p-nitrophenyl phosphate (PNP) substrate (AGDIA, Elkhart, IN) was added to each well and the plates were read on a microplate reader (El 800, Bio-Tek, Winooski, VT). The absorbance due to the accumulation of product

was recorded for each well and the difference between the uninfected and infected was calculated.

Isotyping

Supernatant from hybridoma clones that gave positive ELISA results were concentrated using a Micon B-15 Clinical Sample Concentrator (Millipore, Bedford MA). A glass pasture pipette was used to add 5.0 ml of hybridoma supernatant to a chamber of the B-15 and allowed to decrease in volume to a concentration of ≈25X. Ouchterlony double diffusion technique was used to determine the heavy chain isotype of all antibodies that produced positive ELISA results. A 1% (w/v) solution of agar in deionized water was heated in a microwave oven, plated on a glass slide and allowed to cool and form an agar slab. Wells were punched in the agar slab with one central well and several surrounding wells equidistant from the center well. Reagents from a monoclonal antibody isotyping kit (Sigma) were used with 25.0 μl of an anti-isotype stock placed in the center well with 25.0 μl of 20X concentrated supernatant was added to the outer wells. Slides were made with different anti-isotype reagents: anti-mouse IgG1, anti-mouse IgG2a, anti-mouse IgG2b, anti-mouse IgG3, anti-mouse IgA and anti-mouse IgM. After the initial reagents diffused into the agar, 10.0 μl of PBS was added to each well. The slides were placed in a humidified incubator overnight at 37°C.

Following incubation the slides were placed in room temperature PBS for 48 hours, with several changes of PBS to elute all non-conjugated protein. Each slide was covered with moistened filter paper and placed in a 37°C incubator without humidification to dry completely overnight. Once the agar had completely dried, the slides were stained with a 0.1% Coomassie blue solution. Following staining the slide was rinsed with deionized water and placed in destaining solution (5% (v/v) methanol, 7% (v/v) glacial acetic acid in deionized water) for 48 hours with several changes in destaining solution.

Indirect Immunofluorescence (IF) Assay

OMK monolayers were grown in 35mm dishes on sterilized glass cover slips (Corning, New York). Half of the cover slips were infected with TPV as described and kept in maintenance medium until 95% of the cells demonstrated CPE. The maintenance medium was removed and 100% ethanol at -80°C was added to each dish for 30 seconds to fix the cells. The ethanol was aspirated and the cells were washed with PBS with 0.1% gelatin (BIO-RAD) to block any non-specific protein binding. An uninfected and infected coverslip was covered with 1 ml of 1X hybridoma supernatant and placed in a 37°C humidified incubator for 2 hours. The supernatant was aspirated and the coverslips were washed three times with PBS with 0.1% gelatin. The secondary antibody, anti-mouse IgG fluroescein isothiocyanate

(FITC) conjugate developed in goat (Sigma) was diluted 1:100 in PBS with 0.1% gelatin and then added to each cover slip and incubated in a 37°C humidified incubator for 2 hours. Each cover slip was washed with PBS with 0.1% gelatin to remove any unbound secondary antibody. Each cover slip was mounted on a glass slide with 20% glycerol and sealed with "Nailslicks" (Cover Girl, Hunt Valley, MD). Slides were then viewed on Nikon MICROPHOT-FXA (Nikon, Tokyo, Japan) as phase contrast images and indirect fluorescent (IF) images. For the IF images, the excitation filter set to 450-490 nm and barrier filter set to 520 nm using a super high pressure mercury lamp (model HB-10101AF, Nikon).

SDS-PAGE

Two confluent OMK monolayers in 35 mm dishes were infected with concentrated TPV at a multiplicity of infection (110 pfu/ml). These two dishes along with a mock infected dish were placed on a rocker and adsorption was allowed to take place at 4°C for 90 minutes. After adsorption 2.0 ml of maintenance medium was added to each dish and cytosine arabinoside (Ara-C) was added to a final concentration of 10^{-3} M to one of the virus infected preparations. All preparations were incubated at 37°C and 5% CO_2 for 72 hours. At 72 hours post infection the medium was removed and the cells were washed with 4°C PBS. 100.0 μl of 1% (v/v) Nonidet 40 (NP40) and

100.0 μl of 2X dissociation buffer (0.125M Tris HCl pH 6.8, 4.6% (w/v) sodium dedecyl sulphate (SDS), 20% (w/v) glycerol, 10% β-mercaptoethanol (ME) and 0.002% bromophenol blue). Each preparation was collected in an Eppendorf centrifuge tube and repeatedly pipetted up and down with a hamilton syringe to physically break up the strands of DNA until the preparation was less viscous. Immediately prior to loading, each sample was heated to 100°C for 3 minutes to solubilize all proteins. Twenty μl of each sample along with 5.0 μl of Seeblue protein standard (Invitrogen, Carlsbad, CA) was loaded into a 12% Tris-Glycine gel and ran at 125 volts in an Xcell II mini-cell (Novex, San Diego, CA) for approximately one hour.

Western Blot

After approximately one hour the gel was removed from its cast and transferred to a nitrocellulose membrane using 4°C transfer buffer (running buffer 0.025M Tris-base, 0.192 M glycine, 0.1% (w/v) SDS and 30% (v/v) methanol) and a Trans-Blot SD (BIO-RAD) semi-dry transfer cell. The system was set for one hour at a constant 12 volts. The Western blot was then place in 4°C blocking buffer [Tris buffered saline (TBS) with 5% (w/v) nonfat dry milk (Carnation)] and rocked for 50 minutes at room temperature. The blocking buffer was removed from each blot and 2.0 ml of the appropriate hybridoma supernatant (1x) was added to the blot and sealed in

a kepex bag. Each bag was rocked at room temperature for 50 minutes and stored at 4°C overnight. Each blot was placed in a dish on an orbital shaker and washed for six, approximately 10 minute stages in washing buffer (TBS w/ 0.01% (v/v) tween 20, pH 7.6). Five μl of secondary antibody, anti-mouse IgG peroxidase conjugate developed in goat (Sigma) was added to 2.0 ml of blocking buffer and placed with the blots in clean microscope slide dishes on the orbital shaker for 90 minutes at room temperature. Each blot was removed and placed in a dish on an orbital shaker and washed for six approximately 10 minute stages in washing buffer as before. The o-Phenylenediaimine (OPD) (Sigma) substrate was prepared by adding 6.1 ml 0.1M citric acid, 6.4 ml sodium phosphate, 12.5 ml deionized water and 10.0 μg OPD. Ten μl of 30% (v/v) hydrogen peroxide was added once the OPD was completely dissolved. 3.0 ml of substrate was added to each blot in a darkened room and a digital picture was taken once bands became visible (approximately 5 minutes).

Neutralization Assay

Two volumes of 1x hybridoma supernatant (200.0 μl) were mixed with one volume (100.0 μl) of a 1:100 dilution of supernatant from TPV infected OMK cells. Hybridoma growth medium was added in place of the hybridoma supernatant (200.0 μl) to 100.0 μl of a 1:100 dilution of supernatant from TPV

infected OMK cells as a control. This mixture was incubated at 37°C for 2 hours and 100.0 μl of this mixture was added to a well of a six well dish with confluent OMK monolayers in duplicate. After adsorption at room temperature for 90 minutes, the innoculum was removed and 3.0 ml of overlay medium (maintenance medium with 0.5% (w/v) methyl cellulose) was carefully added. The plates were incubated for 10 days at 37° C and 5% CO_2. The overlay medium was then aspirated from each well and 1ml of 0.1% crystal violet staining solution was added to fix and stain the cells. After 5 minutes the plates were rinsed with water and allowed to dry. Plaques were counted and the average for the wells was used to calculate plaque forming units (pfu)/ml as described by Essani and Dales (1979).

Cloning by Limited Dilution

All hybridoma clones that produced positive ELISA screening results were cloned to ensure the presence of only one clone and decrease the chances of propagating non-producing clones. Positive clones were suspended through pipetting and plated out in serial twofold dilutions with hybridoma growth medium. The group in which about half the wells had no growth was considered to be the progeny of single cells and were rescreened for antibody production (Coligan *et al.*, 1996).

Cryopreservation of Hybridoma Cells

Positive hybridoma clones were grown in 25 cm^2 tissue culture flasks in hybridoma growth medium. Each flask to be frozen was fed the day before freezing to ensure the cells were in exponential growth phase. The cells were resupended by pipetting and transferred to a 15.0 ml conical centrifuge tube and centrifuged (75g Damon IEC HN-SII, 1500 rpm) at room temperature for 2 minutes. The supernatant was removed and replaced with FBS with 8% (v/v) sterile dimethyl sulfoxide (DMSO) (freezing medium). The cells were then placed in freezing vials and placed in a freezing container to achieve a -1°C/min rate of cooling and stored in a -80°C freezer for 24 hours. Following this 24 hour period the cells were immediately transferred into liquid nitrogen for cryopreservation. One week after cryopreservation, one vial was removed from the liquid nitrogen and immediately placed in a 37°C water bath. The thawed cells were diluted 1:20 with hybridoma growth medium in a 25 cm^2 flask and incubated at 37°C and 5% CO_2 to check for viability.

RESULTS

Development of Monoclonal Antibodies Directed Against Tanapoxvirus

Modifications to the technique developed by Kohler and Milstein (1975) as described by Haller (2001) were used to develop hybridoma clones that produce mAbs directed against TPV encoded proteins. Over 3000 wells were initially screened by ELISA. Over the course of three years six fusions were carried out with widely varying results. The first two fusions produced relatively few viable clones and no clones that produced positive results by ELISA were able to be cultivated. The third fusion produced three clones that produced positive ELISA results and were able to be cultivated in 25 cm^2 flasks, but these clones were determined to be of the IgM isotype and deemed of little interest. The next two fusions produced eight clones that produced positive ELISA results and were able to be cultivated in 25 cm^2 flasks, but despite attempts at cloning by limited dilution every culture eventually became non-producing and produced negative ELISA results. The sixth fusion was by far the most productive yielding a dramatic increase in the number of viable clones. Seven clones that produced positive ELISA results were cultivated in 25 cm^2 flasks with six clones being characterized. The six

clones were labeled 1C11, 2G1, 2G3, 3A10, 4A4 and 5A11. The ratio of immunized splenocytes to myeloma cells for the sixth fusion was 1:1, whereas the ratios from the other fusions ranged from 8:1 to 5:1. Established flasks of hybridomas were periodically verified by the original ELISA procedure to ensure antibody production and to guard against the propagation of non-producing clones. Cryopreservation of hybridomas using hybridoma growth medium supplemented with 10-30% DMSO had limited success. Hybridoma viability after removal from liquid nitrogen was slightly improved by diluting the contents of the cryovial 1:20 with hybridoma growth medium and then centrifugation followed by discarding the supernatant and resupending of the pellet in hybridoma growth medium prior to incubation. Residual DMSO from the freezing medium has been reported to have a higher toxicity to murine myeloma cells compared to most cell lines (Freshney, 1994). Hybridoma growth medium supplemented with 10-50% glycerol was also tried with better results. Optimal results for the cryopreservation and retrieval of antibody producing hybridoma cells was obtained by using hybridoma tested FBS (Sigma) with 8% (v/v) DMSO as a freezing medium.

Isotype Characterization of Monoclonal Antibodies

Ouchterlony double diffusion assay was used to determine the isotype of each of the hybridoma clones. A summary of the results is compiled in Table 1.

Table 1.

Isotypes of TPV-Specific Monoclonal Antibodies

Monoclonal antibodies	Isotype
1C11	IgG2a
2G1	IgG1
2G3	IgG1
3A10	IgG1
4A4	IgG1
5A11	IgG1

Isotypes were determined by the Ouchterlony double diffusion method using reagents from a Sigma isotyping kit.

Indirect Immunofluorescence (IF) Assay

All clones that tested positive in the initial ELISA screening demonstrated binding of mAb to TPV infected OMK cells through IF asssays. PBS with 0.1% gelatin was used for each washing cycle. PBS with 0.01% tween was tried as a washing agent, but it resulted in a much higher background level. The IF images show mAb binding in localized areas of the cytoplasm of TPV infected cells (Figures 1a and 1b). These areas of intense

FITC staining indicate the location of "Viral factories", a term used by Fenner (1996) to describe the centers of viral replication in the cytoplasm of poxvirus infected cells. Phase Contrast (PC) images corresponding to each IF image clearly show the borders of all cells for reference. Indirect fluorescence images of uninfected OMK cells treated with hybridoma mAb and the anti-mouse IgG FITC conjugate were indistinguishable from uninfected and infected OMK cells treated with anti-mouse IgG FITC conjugate only.

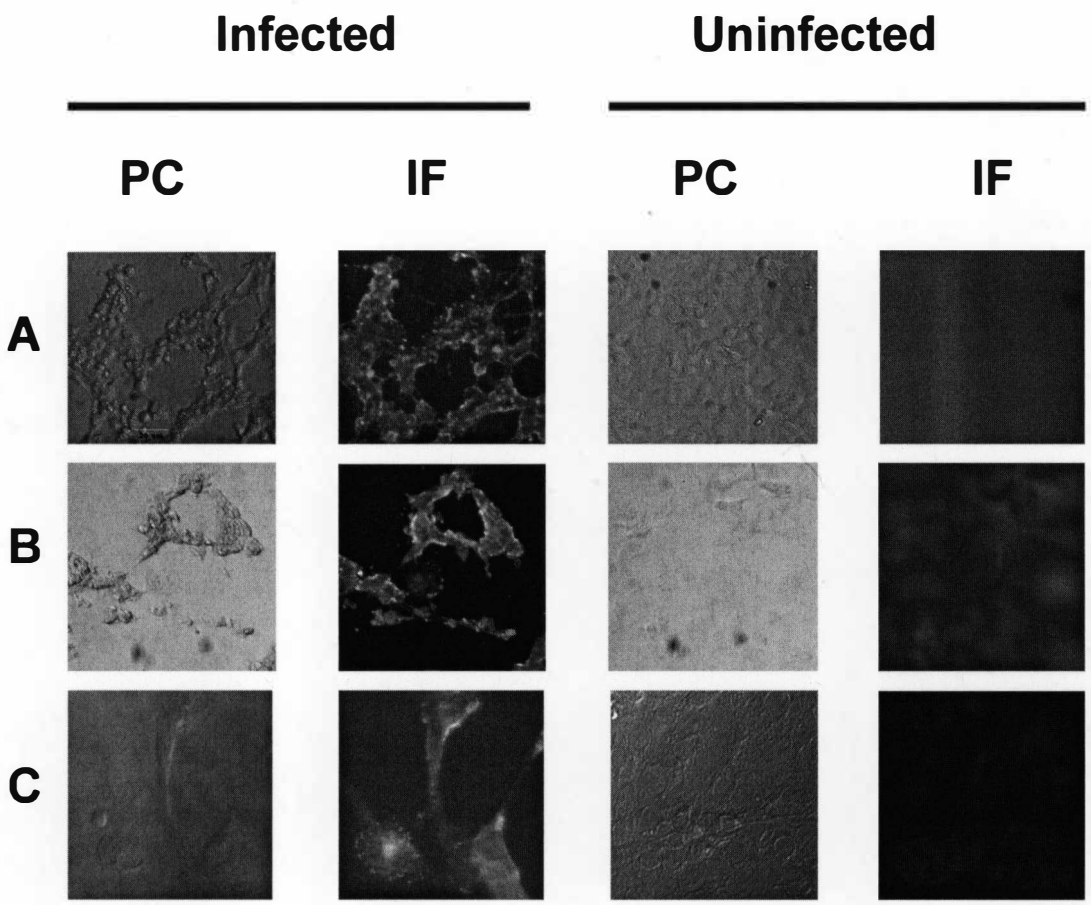

Figure 1a.

Indirect Immunofluorescence Microscopy Demonstrating Monoclonal Antibody Binding to TPV Proteins in Infected Cells. OMK cell monolayers were infected with TPV and ethanol fixed, or mock infected and ethanol fixed. All monolayers were treated with murine hybridoma supernatant, washed and then treated with anti-mouse IgG FITC conjugate. Phase contrast (PC) and Indirect Immunofluorescence (IF) images were taken of each. Each row consists of images taken of monolayers treated with the same hybridoma supernatant. The rows are designated as A; 1C11, B; 2G1, C; 2G3. Bar = 50mm

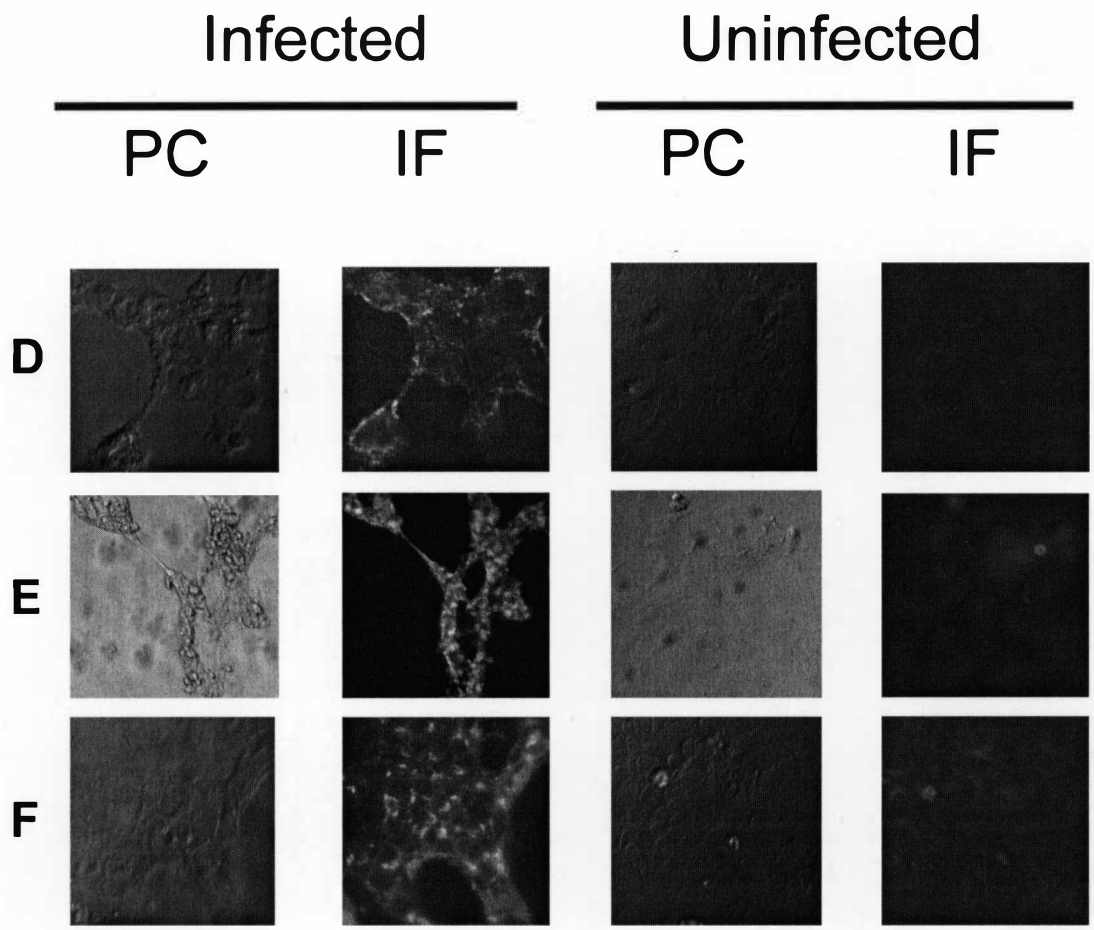

Figure 1b.

Indirect Immunofluorescence Microscopy Demonstrating Monoclonal Antibody Binding to TPV Proteins in Infected Cells. OMK cell monolayers were infected with TPV and ethanol fixed, or mock infected and ethanol fixed. All monolayers were treated with murine hybridoma supernatant, washed and then treated with anti-mouse IgG FITC conjugate. Phase contrast (PC) and Indirect Immunofluorescence (IF) images were taken of each. Each row consists of images taken of monolayers treated with the same hybridoma supernatant. The rows are designated as D; 3A10, E; 4A4, F; 5A11. Bar = 50mm

2G1, 2G3, 3A10, 4A4 and 5A11 mAbs Bind to Late Viral Proteins

Poxvirus infection causes the production of viral proteins by the host cell that differ in the time of their initial production and level of their production over the course of the infection (Moss, 1996). By comparing mAb binding to proteins from TPV infected OMK cells versus binding to proteins from uninfected OMK cells the specificity of mAb to viral proteins can be determined. By comparing mAb binding to proteins from TPV infected OMK cells versus binding to proteins from TPV infected OMK cells that have been treated with inhibitor of DNA synthesis, Ara-C, the viral induced protein of interest can be characterized as an early or late viral protein. If binding occurs only to proteins from TPV infected OMK cells that have not been treated with Ara-C, that protein would be considered a late protein because DNA synthesis is required for the production of that protein. If binding only occurs to proteins from TPV infected OMK cells treated with Ara-C, that protein would be considered a late protein. If binding occurs to the same protein from TPV infected OMK cells regardless if treatment with Ara-C occurred of not, that protein would be considered constitutively expressed throughout infection.

Proteins from uninfected OMK cells, TPV infected OMK cells and Ara-C treated OMK cells infected with TPV were separated by SDS PAGE and then transferred to a nitrocellulose membrane, creating a western blot. mAb

binding was identified by ELISA. Hybridoma clones 2G1, 2G3, 3A10, 4A4 and 5A11 all demonstrated binding to late viral proteins with 2G1, 2G3 and 5A11 showing binding to two separate late proteins (figure 2.). The apparent molecular mass of the proteins bound by each mAb was estimated by the method established by Laemmli (1970). Hybridoma clone 1C11 did not demonstrate any observable specific binding despite numerous positive screening ELISA results with anti-mouse alkaline phosphate conjugate (Sigma) as the secondary antibody (Table 2.).

Table 2.

Monoclonal Antibodies and the Viral Polypeptides They Recognize

Monoclonal Antibody	Apparent Molecular Mass
1C11	-*
2G1	16kDa 28kDa
2G3	21kDa 30kDa
3A10	18kDa
4A4	38kDa
5A11	12kDa 36kDa

* Monoclonal antibody 1C11 reacted in immunofluorescence reactions with TPV infected cells, but not with mock infected cells (see figure 1a).

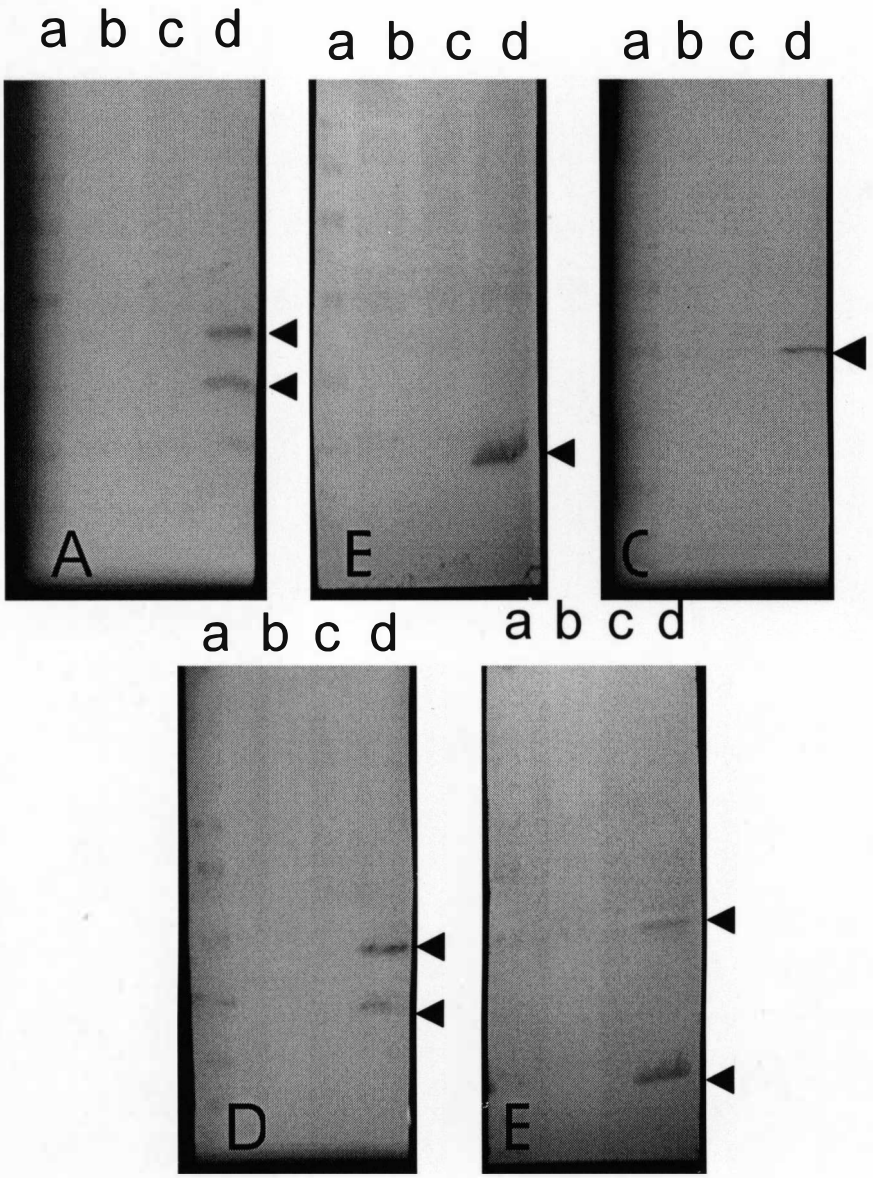

Figure 2.

Western Blot Demonstrating Monoclonal Antibody Binding to Late TPV Proteins. OMK cell monolayers were either mock infected, infected with TPV and treated with Ara-C, or infected with TPV. Cells were harvested and plasma membranes lysed with NP-40 and dissociation buffer. Sample were loaded into SDS PAGE as follows: (a) Seeblue protein standard with molecule masses of 250, 148, 98, 64, 50, 36, 22, 16, 6 and 4 kDa (b) mock infected OMK (c) TPV infected OMK with Ara-C (d) TPV infected OMK. The proteins in the gel were then transferred to a nitrocellulose membrane creating a western blot. The blots were treated with hybridoma supernatant as follows: (A) 2G1 (B) 2G3 (C) 3A10 (D) 4A4 (E) 5A11. The western blots were then treated with anti-mouse IgG peroxidase conjugate and exposed to the OPD substrate. Arrows were added to the right of each image to indicate the bands of precipitate that were formed.

Neutralization Assay

If a mAb binds to a structural protein on the surface of a virion, viral attachment to the host cell may be inhibited. Supernatant from TPV infected OMK cells was mixed supernatant from each hybridoma clone and compared to the supernatant from TPV infected OMK cells mixed with hybridoma growth medium. If a mAb demonstrates neutralization activity, the number of plaques formed would be decreased relative to the control. Hybridoma clones 2G1 and 5A11 show neutralization activity. The other clones show either slight, or no reduction in pfu/ml (Table 3.). This data is preliminary and the sample size consisted of only two groups, therefore the results are not valid in statistical terms.

Table 3.

Neutralization Assays

Antibody	Titer (pfu/ml)		% Reduction
	with antibody	without antibody	
1C11	780 ± 150	840 ± 0	7
2G1	645 ± 45	840 ± 0	23
2G3	870 ± 30	840 ± 0	0
3A10	780 ± 30	840 ± 0	7
4A4	870 ± 30	840 ± 0	0
5A11	525 ± 15	840 ± 0	38

Two volumes of 1x hybridoma supernatant (200 μl) were mixed with one volume (100μl) of a 1:100 dilution of supernatant from TPV infected OMK cells, incubated at 37° C for 2 hours, and 100 μl of this mixture was absorbed on OMK monolayers in duplicate. Controls were treated in an identical manner, except that growth medium replaced hybridoma supernatant. Virus titer was computed as described by Essani and Dales (1970).

DISCUSSION

The results of this project show five mAbs that bind late viral induced protein. The importance of cloning by limited dilution to isolate and maintain selected hybridomas as a culture that produces mAb cannot be overestimated. A quote from James Goding explains this very well:

> Hybridoma lines should be cloned at least *twice* to make absolutely certain that each is a true clone because the Poison distribution specifies only probabilities not certainties...Repeated subcloning is also important because of the relatively high probability of growth of nonproducer variants owing to chromosome loss. After two cycles of cloning, the rate of chromosome loss is small, although the risk of overgrowth by nonproducer cell is never completely eliminated.

The speed at which a nonproducing variant can overtake a culture was demonstrated by the absence from the data presented here of the seventh clone, 1H6, that was repeatedly screened by ELISA and grown into 75cm^2 flasks. Hybridoma clone 1H6 was cloned by limited dilution, and a well with one small colony that tested positive by ELISA was propagated into several flasks. Once the flasks propagated from this single colony reached an acceptable cell density the original flasks from which the cells were cloned were discarded in favor of the flasks that had a higher probability of

containing a single clone. Two weeks later, each of these flasks either screened negatively, or weekly positive by ELISA during a periodic check of cultures. Isolation of an antibody producing clone from this mixture has not been accomplished at the time of writing this manuscript. It has been suggested that nonproducing hybridoma clones can devote all of their metabolic energy towards cell division, whereas the antibody secreting clones devote much of their energy towards making antibodies and divide at a much slower rate.

Hybridoma clone 1C11 did not demonstrate any specific binding to any specific viral protein, early or late despite numerous positive screening ELISA results with anti-mouse alkaline phosphate conjugate (Sigma) as the secondary antibody. Clone 1C11 also consistently demonstrated the most prominent precipitate during the Ouchtolony double diffusion assays of all clones tested indicating copious amounts of antibody produced. One explanation for this is that the epitope that mAb 1C11 recognizes in the screening ELISA is disrupted by the denaturing environment within the gel during SDS PAGE. This could be tested by immunoprecipitation of viral antigen by Hybridoma 1C11 supernatant prior to electrophoresis.

Hybridoma clones 4A4 and 3A10 each demonstrated binding to a single late viral protein, while clones 2G1, 2G3 and 5A11 each demonstrated mAb binding to two distinct late viral proteins. A mAb generated against YLDV and showing cross reactivity to TPV has also demonstrated binding to

two separate proteins when the immunoprecipitates created by mixing YLDV infected cells with concentrated mAb were separated by gel electrophoresis (Haller, 2001). The presence of two bands being bound by mAb could be explained by possible cleavage of a precursor protein, with both the precursor and cleaved proteins containing the same epitope and thus being recognized by the same mAb. This theory could be tested by comparing mAb binding to several samples of protein from TPV infected cells collected at sequential post infection intervals separated by SDS PAGE and transferred to a western blot. A pulse-chase experiment supports this theory. In this experiment infected OMK cells are exposed to radioactively labeled methionine 24 hours after infection for one hour and then all radioactively labeled methionine that have not been taken up by the infected cells are removed. This is the pulse part of the experiment. Cells are harvested from sequential time periods post pulse and the proteins were separated through SDS PAGE. The change in the profile of the proteins produced by the virally infected cells can be observed over time. The results show many bands are consistently expressed over the course of infection, while some bands decreased over time and some bands increased, or made their first appearance in time periods after the pulse. Since no new radioactive amino acids are available to be incorporated into newly generated proteins, the changes can be attributed to cleavage of existing radioactive proteins explaining the disappearance of other bands. Monoclonal antibody 2G3 bound to two

proteins with the apparent molecular masses of 21 kDa and 30 kDa and there are two proteins of approximately corresponding masses that show changes over time in the pulse-chase experiment [Figure 3., (a) and (c)]. mAb 2G1 bound two proteins with the apparent molecular masses of 16 kDa and 28 kDa. A protein of approximately (BIO-RAD, Richmond, CA) 28 kDa shows a change over time in the pulse-chase experiment [(figure 3., (b)], but a protein of 16 kDa would have been run off the gel used in the pulse-chase experiment presented here. mAb 5A11 binds proteins of apparent molecular masses of 12 kDa and 36 kDa. A protein of 12 kDa would not be contained in the gel used in the pulse-chase experiment, but several proteins around the approximate mass of 36 kDa show a change over time in the pulse-chase experiment [figure 3., (d)]. A protein of 12 kDa would be run off the gel used in this pulse-chase experiment. Other published pulse-chase experiments done with vaccinia virus have shown that several late vaccinia virus proteins undergo cleavage and processing. Some of these late proteins that are processed have been shown to be major structural proteins. No processing of early vaccina virus proteins was identified (Moss and Rosenblum, 1973). The data from the pulse-chase experiment shows a correlation with the results obtained, but further verification is necessary.

There are many potential uses for these antibodies. Clinical use in the diagnosis of TPV is possible. There are many possible applications for these antibodies as basic research tools for the study of TPV and possibly YLDV.

The study of the structure and function of the viral proteins that are bound by these mAbs can be greatly accelerated due to the creation of these Hybridomas. One particularly exciting possibility is the mAb secreted by hybridoma 4A4 which binds a TPV protein with an apparent molecular mass of 30 kDa. A TPV encoded secreted protein with a molecular mass of 30 kDa, which acts as an inhibitor of human tumor necrosis factor has been reported (Brunetti *et al.*, 2003). The mAbs generated by this project may help elucidate the mechanisms of virus-host interactions and possibly the intricacies of the human immune system.

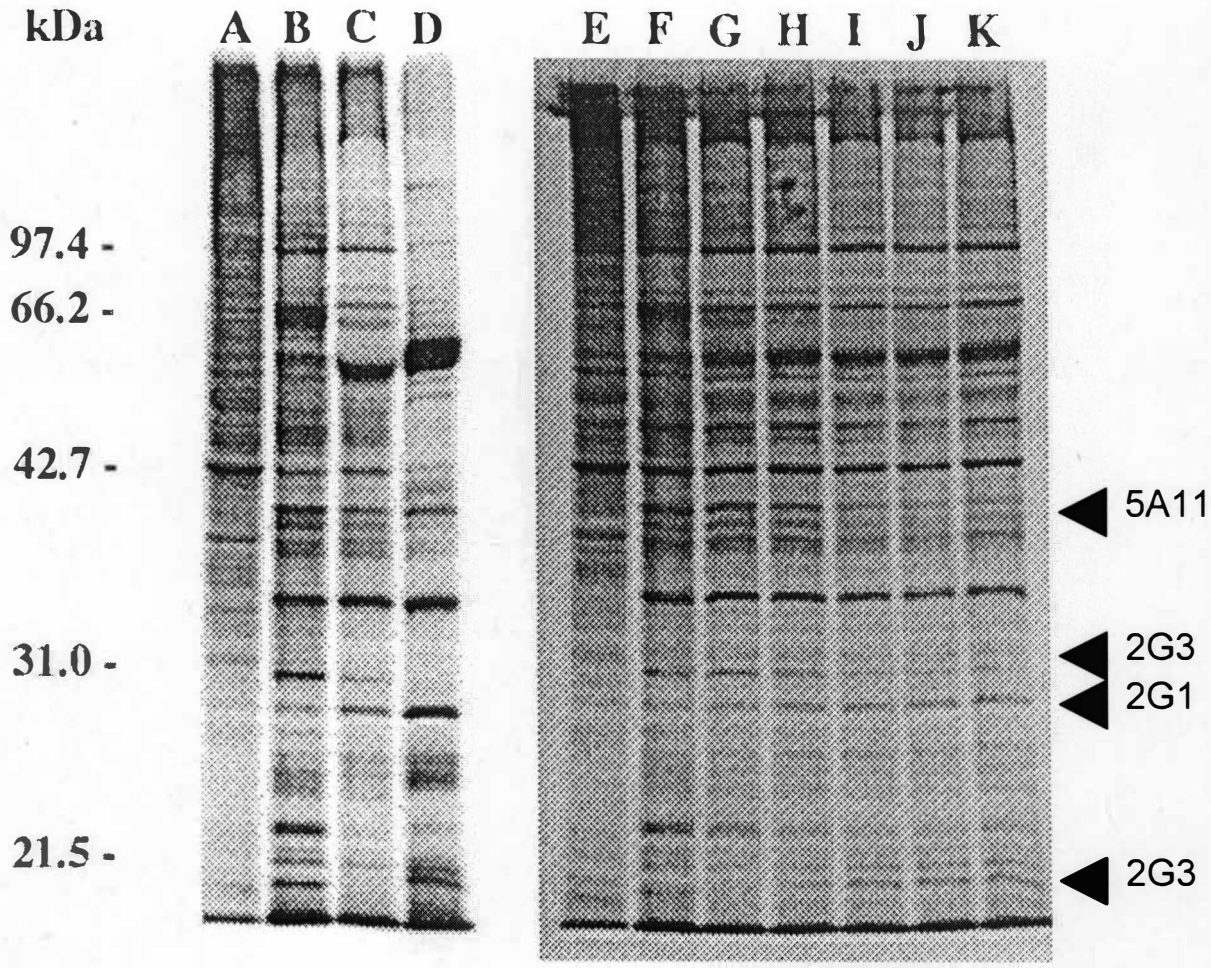

Figure 3.

Post-translational Cleavage of TPV Proteins. The samples in the lanes are described as follows: (A) uninfected OMK cells, (B) infected OMK cells pulsed 18hrs hours post infection (hpi) for one hour (C) infected OMK cells at 72 hrs hpi (chase period), (D) TPV isolated at 72 hrs, (E) uninfected OMK cells, (F) same as B, (G-K) infected OMK cells chased every 6 hrs after pulse. Each arrow designates a protein that changes in concentration over the chase period that correlates to the apparent molecular mass of a TPV protein identified by mAb binding. The approximate molecular mass of each recognized protein is listed as follows: 2G3, 21 kDa and 30 kDa; 2G1, 28 kDa; 5A11, 36 kDa

Appendix

IACUC Approval Letter

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial
1903-2003 Celebration

Date: May 5, 2003

To: Karim Essani, Principal Investigator
Michael Franz, Student Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 03-01-01

Your protocol entitled "Preparation of Monoclonal Antibodies" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: May 5, 2004

Walwood Hall, Kalamazoo MI 49008-5456
PHONE: (616) 387-8293 FAX: (616) 387-8276

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