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Development and use of a monoclonal antibody-based enzyme immunoassay for the detection of maize dwarf mosiac virus

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by

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LITERATURE REVIEW

Maize Dwarf Mosaic Virus

In 1964, Dale (24) isolated a mechanically transmissible phytopathological agent which ellicited mosaic symptoms on corn (Zea mays) and sorghum (Sorghum bicolor). The causative agent, identified as a virus, was designated maize dwarf mosaic virus strain A (MDMV-A) and is now considered to be the most widely occurring viral pathogen of corn in the United States (122, 123). MDMV-A can be distinguished from closely related sugarcane mosaic virus (SCMV) strains by the ability of the former to replicate in Johnsongrass (Sorghum halepense) but not sugarcane (Saccharum spp.). Today, most researchers use the virus designation MDMV; however, some refer to MDMV-A as the Johnsongrass strain of SCMV or SCMV-Jg (110, 138). In 1966, MacKenzie et al. (91) isolated a new strain of MDMV from corn which did not replicate in Johnsongrass. This strain was designated MDMV-B. Other strains of MDMV include MDMV-C, MDMV-D, MDMV-E, MDMV-F, and MDMV-O (90, 94); however, only MDMV-A and MDMV-B are considered to be economically important pathogens of corn and sorghum (141).

MDMV is a member of the largest group of plant

viruses, the potato virus Y group. Members of this group have characteristic flexuous filamentous particles, normally 720-900 nanometers (nm) long and 11 nm in diameter (47, 57). The genome consists of a single stranded, positive sense, ribonucleic acid (RNA) molecule, with a molecular weight of 3.0-3.5 megadaltons (47, 52, 54, 57). The RNA genome is enclosed within the particle by up to 2000 repeating subunits of a single protein species (molecular weight 3.0-3.7 kilodaltons) arranged in a helix (47, 51, 54, 57, 83). Definitive members of the potato virus Y group are transmitted non-persistently by aphids and induce the formation of characteristic cytoplasmic inclusions (21, 57, 68, 95). Cytoplasmic inclusions are unique to the potato virus Y group and can be useful for the diagnosis of infection at the group level (33, 47).

MDMV is an important phytopathological agent of maize where it can potentially reduce corn grain yields by as much as 45% (122). Symptoms induced by MDMV in maize include local and systemic necrosis, systemic mosaic, dwarfing, and occasionally leaf reddening (54, 109, 110, 111). The development of various symptom patterns depend upon host genotype (11, 111, 139), virus strain (34, 109, 121), time of inoculation (63, 122), and certain environmental factors including temperature (45, 67, 129) and light (67, 129). Infected plants also exhibit numerous

physiological and biochemical changes including the formation of viral coded cytoplasmic inclusions (21, 57, 68, 95), unusual aggregates of mitochondria and chloroplasts (57), reduction in transpiration rate (88), decreased ear diameter, length, and width, (20, 97), sterility expressed as missing kernels (98), stunting (109), reduction of stalk diameter and strength (73), and a reduction in seed quantity and quality (20, 97).

MDMV has an extensive host range, infecting more than 200 members of the Gramineae (120, 141). Of the two major strains used in this study, strain A has a wider geographical range and taxonomic distribution within the Gramineae than strain B (120, 121, 123). Furthermore, many grasses which are hosts to both strains produce less severe symptoms when infected with MDMV-B (121). Within the Gramineae, Johnsongrass has been recognized as the principal reservoir for MDMV-A survival. In addition, Knoke et al. (76) have shown that infected Johnsongrass is the main virus source for initial vector acquisition and subsequent spread to maize.

The means by which MDMV-B overwinters is still unknown. The virus has a limited perennial grass host range; none of these species have been shown to harbor MDMV-B. Evidence does exist for a low incidence of MDMV-B transmission through corn seed (56, 61, 130); thus, it

would seem entirely possible that seed transmission in wild grasses could serve as the primary virus source.

MDMV can be mechanically transmitted; however, aphid vectors seem to be the major source of spread within the field. As with all members of the potato virus Y group, MDMV is transmitted by aphids in a non-persistent or stylet-borne manner. Acquisition of MDMV occurs when aphids alight on a leaf surface and make brief stylet probes (usually less than 30 seconds) into the leaf epidermal cells. These probes are presumably to test the suitability of the leaf as a food source. Upon regurgitation of the plant sap the virus absorbs to the dry stylet surface (96). Once acquired, the virus can be transmitted immediately to one or only a few healthy plants during subsequent inoculation probes (93). Viruses transmitted non-persistently cannot multiply or circulate within their vectors. Therefore, the ability of viruliferous aphids to transmit non-persistent viruses typically persists for only 1-4 hours following acquisition. However, Berger (8), has shown that the MDMV vector Schizaphis graminum can remain viruliferous for up to 21 hours following acquisition. Due to the stylet-borne mode of transmission, MDMV has a low level of vector specificity and therefore can be transmitted by at least 26 aphid species (75, 116, 141). MDMV-B has also been shown to be trans-

mitted through soil; however, the soil inhabiting vectors have not been identified (12).

The extent of an MDMV epiphytotic is dependent upon the number and proximity of primary virus sources, the gradation of aphid vector populations, and the susceptibility or resistance of the maize hybrids grown in the region (162). Therefore, control of MDMV can be attempted in a variety of ways. Eradication of the primary virus source would serve as one method of control. However, as noted previously, the primary source of MDMV-B is not known. In addition, the large perennial host range of MDMV-A would make eradication an expensive and incomplete control measure.

Control of vectors is another possible way to limit virus spread. This usually involves the reduction of aphid populations with systemic insecticides. Although a viruliferous aphid will be killed by a systemic insecticide, MDMV can be transmitted in a single leaf probe. The use of leaf oils, which inhibit aphid probing behavior, have been suggested for the control of non-persistent transmitted viruses. However this method is expensive and short-lived (141).

Undoubtedly the most successful and widespread control method for MDMV is breeding for resistance or tolerance. This process involves the selection of resis-

tance or tolerance genes and the subsequent breeding of these genes into a cultivar genome which exhibits acceptable agronomic characteristics. Although tolerance and resistance are the control methods of choice, each has its limitations. Infected tolerant plants allow the replication of virus; however, symptoms are suppressed. This may not be an acceptable solution, for tolerant plants may serve as a reservoir for the spread of virus to other hosts (93). Resistant plants do not allow the replication of virus; however, Findley et al. (34) and Presley et al. (111), have shown that resistance to MDMV is strain specific. Furthermore, almost all corn inbred lines which demonstrate varying degrees of resistance to MDMV-A are highly susceptible to MDMV-B (121). Because of this inherent problem, it would be desirable to index a maize plot and determine if a "resistant" host is infected with one or more strains of MDMV.

Several methods exist for differentiating the two major strains of MDMV. Use of differential hosts (corn inbred line E663 for MDMV-A and Setaria adhaerans for MDMV-B) has been suggested by Rosenkranz (119) and Tosic and Ford (144). However the use of these hosts is impractical. Only a small percentage of MDMV-B inoculated hosts will produce symptoms and symptom development for both viruses may take 7-14 days (121). In addition,

valuable greenhouse space must be expended to harbor the assay plants. The problems inherent with biological assays can be alleviated through the use of immunological assays. These assays have the potential for very high sensitivity and strain specificity. Furthermore, immunological assays could easily accommodate the indexing of numerous samples in a relatively short period of time. On the other hand, immunological assays detect antigenic sites on the virus; which may not be an accurate measurement of infectious viral particles.

Application Of Immunoassays To Plant Virology

Plant virologists have made increasing use of serological methods as a sensitive and specific tool for the indexing of plant viruses. When viral antigen combines with specific antibody, a precipitate forms which is visible with the unaided eye or a binocular microscope. The extent of precipitation is dependent upon a number of factors including salt concentration, pH, temperature, and, most importantly, the ratio of concentration of antibody and viral antigen. If excess of either antibody or antigen exists, inhibition of precipitation may occur. The concentration of antiserum and antigen which results in the optimal precipitation is termed the concentration

of equivalence for that specific antiserum and virus system. Some of the more commonly utilized immunological assays based upon the precipitation reaction include microprecipitin (153), passive hemagglutination (125), and Ouchterlony gel diffusion (105).

The microprecipitin test is primarily utilized for determining specific antiserum titer and the concentration of equivalence. The reaction of a virus with a specific antiserum is clearly visualized if a series of twofold dilutions of both reactants are mixed in all combinations. The inverse of the highest antiserum dilution which develops a visible precipitate is termed the titer of that antiserum. The concentration of equivalence occurs at the antiserum and virus dilution which elicits the first visible precipitate.

Passive hemagglutination is a modified form of the microprecipitin test. Viral antigen or specific antiserum are non-specifically adsorbed to sensitized sheep red blood cells (SRBC). With the addition of small amounts of the other reactant, the sensitized SRBC form a large, easily visible precipitate. The usefulness of microprecipitin and hemagglutination assays as indexing tools is limited due to the non-specific aggregation of plant chloroplasts and other cellular debris in the liquid medium (2). These problems can be eliminated by performing

the precipitation reaction in a gel medium.

Since its introduction in 1962, Ouchterlony gel diffusion has been one of the more popular techniques utilized to assay plant viruses. Briefly, wells are formed in some defined geometrical pattern in a thin layer of agar. The specific antiserum is usually placed in a central well and the viral antigen solutions in surrounding wells. Virus and antiserum diffuse towards one another through the agar and form a precipitation line where the two reactants are present in optimal proportions. The formation and position of a precipitation line is completely dependent upon a balanced system of virus and antiserum (6). Therefore, one must determine the concentration of equivalence for each antibody-antigen system studied. An optimized assay can detect 1-2 ug/ml of virus (38) and has been the basis of several commercial screening programs (26, 93). In addition, the Ouchterlony double diffusion assay has been used to determine serological relationships between closely related viruses (1, 99). Problems associated with the use of gel diffusion assays include viral aggregation resulting in artifacts and the diffusion of rod shaped particles, such as MDMV, may be restricted due to particle shape. Degradation of viral particles prior to diffusion has been suggested; however, this process may destroy viral epitopes

(6, 55, 93).

For many important plant viruses, serological techniques performed in liquid or gel media cannot be utilized because of certain limitations such as low virus concentration, unsuitable particle morphology, or the presence of aggregating cellular debris in cell extracts. These problems can be eliminated through the use of solid-phase supports, which immobilize reagents to a solid surface. These assays are based upon the observation that proteins will hydrophobically interact and nonspecifically bind to plastic surfaces (86). Some of the most commonly used solid-phase assays include serologically specific electron microscopy (7), enzyme-linked immunosorbent assay (22), radioimmunoassay (5), and, most recently, dot immunobinding assay (49).

Serologically specific electron microscopy (SSEM) was first introduced by Ball and Brakke (7). The original procedure has been modified in various ways to produce a rapid diagnostic test (106). Briefly, electron microscopy grids coated with a plastic membrane are floated on specific antiserum. Unbound antiserum is removed by washing and the grid is immediately floated on 50 ul drops of infected tissue extracts. Viral particles immobilized on the grid are then stained and observed under the electron microscope. SSEM was found to be much more

sensitive than gel diffusion and, in some cases, more sensitive than enzyme-linked immunosorbent assay (48). Furthermore, Alexander and Toler (3) have coupled SSEM with immuno-gold decoration to differentially label viral particles of MDMV-A and MDMV-B in a mixed preparation. Although SSEM is very sensitive and highly specific, the technique requires considerable training, expensive equipment, and is time consuming. Therefore, this technique would not be well-suited for the routine analysis of a large number of samples.

In 1977, Clark and Adams (22) showed that enzyme-linked immunosorbent assay (ELISA) could very efficiently be applied to the rapid and sensitive detection of plant viruses. This technique couples the use of a polystyrene solid-phase with specific antibody conjugated with enzyme. For plant viruses, the double-sandwich form of ELISA has been found to be suitable (155). In this method, specific antibody adsorbed to a polystyrene microtitration plate selectively traps and immobilizes virus in extracted tissue. Captured virus is further reacted with specific antibody to which an enzyme has been conjugated. After washing, enzyme conjugated antibody bound to virus is detected colorimetrically by the addition of a suitable enzyme substrate.

Generally, the system described above can detect

virus at concentrations of 10-100 ng/ml (89, 155); however, Clark and Adams (22) have reported detection levels below 1 ng/ml. This method can easily be applied to the indexing of a large number of samples in a relatively short period of time. For example, the double-sandwich ELISA (ds-ELISA) has been used to assay individual soybean seeds and leaf extracts for the presence of tobacco mosaic virus and soybean mosaic virus (53, 89). In addition, Sum et al. (137) have shown that ELISA was 100 times more sensitive than infectivity tests for the detection of MDMV. However, one major limitation has been encountered with the ELISA technique.

Koenig (77) reported that the binding of enzyme conjugated antibodies in an ELISA was completely inhibited by simultaneously added native antibodies. She suggested that the harsh conjugation procedure may alter the antibody molecule or the enzyme may allosterically hinder the binding sites; thus, reducing the binding abilities of the antibodies. In some cases the enzyme conjugation procedure may reduce antibody binding by 60-88 % (40). This problem can be circumvented through the use of solid-phase radioimmunosorbent assay (RIA) with radiolabelled antibody (40, 62).

The first RIA used for the detection of plant viruses was developed by Ball in 1973 (5). Her assay was based

upon the ability of sample antigen to compete with labelled antigen for binding to a limited number of antibody molecules. Briefly, specific antibody is irreversible adsorbed to the walls of polystyrene disposable centrifuge tubes. A specified amount of radiolabelled virus is added simultaneously with tissue extract containing an unknown amount of unlabelled virus. The amount of captured labelled virus can be measured and is inversely proportional to the amount of virus in the test sample. This assay is fairly sensitive and specific; however, it requires the labeling of purified virus. Therefore, use of such a system with viruses that are difficult to purify would not be economically feasible.

In 1980, Ghabrial and Shepherd (40) developed a microtitration plate RIA based upon the principal of the double-antibody sandwich described previously. Their RIA required the radioiodination of purified antiserum rather than viral antigen. Using polystyrene microtitration plates as the solid-phase, this assay was performed much like the ds-ELISA of Clark and Adams (22); however, addition of enzyme-conjugated antibody is replaced by radiolabelled antibody. This assay requires an additional step as bound radiolabelled antibody must be disassociated from the virus by acidification. The released radioactivity is then measured in a scintillation counter. This

RIA was 10 times more sensitive than ELISA when detecting the presence of lettuce mosaic virus in tissue extracts (40). Therefore, RIA offers an alternative to ELISA for systems where the latter gives borderline or inconclusive results due to insufficient sensitivity. Furthermore, in 1983 Bryant et al. (17) designed a solid-phase double antibody sandwich RIA which utilizes antibody coated polystyrene beads rather than polystyrene microtitration plates. Therefore, to measure the bound labelled antibody one simply places the entire bead into a scintillation vial with 5.0 ml of Riafluor scintillation cocktail. This assay eliminates the need to disassociate and isolate bound labelled antibody from the virus. Thus the time required to perform this RIA is the same as a ds-ELISA. In addition, recent tritium labelling procedures provide a simple, gentle, and efficient procedure for radiolabelling antibodies without loss of biological activity (69). On the other hand, the use of ELISA has certain advantages over RIA including economic preparation and long shelf life of enzyme conjugated reagents, freedom from radiological safety procedures, and results which can be scored visually or with simple inexpensive equipment.

Most recently, Hibi and Saito (49) have developed a sensitive dot immunobinding assay (DIBA) based upon the immunobinding techniques of Towbin et al. (147) and the

principles of ds-ELISA. This assay is performed much like a ds-ELISA. However, nitrocellulose filter paper is utilized as the solid-phase support rather than polystyrene and the product of the enzyme reaction is insoluble. Using their procedures, Hibi and Saito (49) could complete a DIBA in 4-5 hours and detect 1 ng/ml of tobacco mosaic virus in tissue extracts. However, at low tissue dilutions they observed high non-specific reactions with plant extracts. These non-specific reactions persisted when antiserum cross adsorbed with plant tissue was used. Therefore, this technique would not be useful for detecting the presence of viruses which typically occur at low concentrations in their host. Although some technical improvements are necessary to reduce non-specific reactions, this assay may prove to be a simple more rapid test than ELISA for indexing tissue extracts.

Antiserum typically utilized in immunoassays consists of a heterogenous population of antibody molecules. The characteristics of a particular antiserum are dependent upon the affinity, avidity, and specificity of each antibody molecule within the mixed population. Therefore, specific antiserum derived against one antigen could very possibly contain antibody molecules which will cross-react with a closely related heterologous antigen. This occurs if the antigens possess certain conserved epitopes which

are recognized by the immunized animal as common to both. This situation exists with strains A and B of MDMV (64, 109, 133). Therefore, an immunoassay developed using antiserum derived against MDMV-A, will not only detect the presence of MDMV-A but will also detect the heterologous strain MDMV-B. Normally this would not be a problem; however, Pioneer Hi-Bred International, Inc. has expressed interest in a rapid, strain specific, immunoassay for the detection of MDMV to be utilized in their breeding program.

The majority of antibodies specific for conserved viral epitopes can be removed through extensive cross-absorbtion with the heterologous strain; however with their great sensitivity, most immunoassays can detect even very small amounts of cross-reacting antibody. Alternatively, problems inherent with conventional heterogenous antiserum can be eliminated through the use of monoclonal antibodies (McAbs).

The clonal selection theory proposed by Burnet (18) states that an antibody-producing B-cell is committed to generating homogenous antibody molecules with the same affinity, avidity, and specificity. A B-cell can become cancerous like any other cell. The result is a single cell myeloma whose clones secrete a homogenous population of antibody molecules or McAbs. The induction of myelomas

is a completely random process; therefore, it would be difficult to isolate a McAb producing myeloma specific for a particular antigen.

In 1975, Kohler and Milstein (79) developed a method to solve this dilemma. They fused an antibody secreting spleen cell from an immunized animal with a myeloma cell. The result is an immortal "hybridoma" which secretes a McAb of predetermined specificity, characteristic of the spleen cell. Using this technique, cell lines producing McAbs of almost any specificity can be obtained in virtually unlimited amounts.

The use of McAbs in the biomedical field is well established; however McAb production has only recently been applied to the field of plant virology (59). Over the past four years, plant virologists have utilized McAbs to improve already existing, well-established immunological techniques including diagnosis of viral infection (32, 92), virus strain differentiation (14), demonstration of serological relationships (31), and quantitation of viral antigens (58). In addition, techniques unique to McAb methodology have been developed including virus purification by affinity chromotography (30), epitope specific neutralization of virus infectivity (60), and molecular analysis of coat protein epitopes (153). The use of hybridoma technology provides a powerful research tool for

the plant virologist; however, certain limitations have been encountered.

McAbs, as all antibody molecules, are heterospecific and may react with greater affinity with antigens other than the original immunogen (10, 19). Heterospecificity may occur between closely related or completely unrelated antigens. This type of cross-reactivity is indicative of a shared conformational or sequential epitope recognized by the McAb. Heterospecificty is usually not observed simply because the investigator does not look for the unrelated cross-reacting antigen.

McAbs, being a homogenous population, contain only a subset of the properties observed with conventional polyclonal antisera. Therefore, McAbs cannot necessarily be utilized in assays developed with polyclonal antisera. Many McAbs are specific for epitopes which are inaccessible on the antigen surface. This reduces the possibility of antibody-antigen cross-linking and thus precipitation formation (72). Therefore, McAbs which possess these characteristics cannot be used in assays which require visible immunoprecipitation, such as Ouchterlony double diffusion and microprecipitin tests. Additional problems have been associated with immunoassays such as ELISA and RIA which utilize antibody labelled with enzyme or radioisotope, respectively. Many McAbs are

extremely labile and labelling may result in loss of biological activity or alteration of specificity (60). Furthermore, loss of activity may occur when an unstable McAb is bound to a solid-phase support (148). To obtain a McAb with the desired characteristics, one should screen the McAb producing hybridomas utilizing techniques for which the McAb will ultimately be intended.

MATERIALS AND METHODS

General Methods

Distilled and deionized water

Reagent grade 1 distilled-deionized water (ddH_2O) was used in the preparation of all media and buffers and as the final rinse for all glassware and plasticware. This was obtained by passing distilled water through a Super-Q Ultra Pure Water System (Millipore Corp., Bedford, MA) containing activated charcoal, which removes organic ions, and a mixed bed ion-exchange resin, for removal of inorganic ions.

Phosphate buffer

A solution of 0.05M $NaKPO_4$, pH 7.6, was used as a diluent when grinding MDMV infected tissue for virus inoculum. This buffer was prepared as follows:

Solution A:

Na_2HPO_4 $7H_2O$	13.4 g
ddH_2O up to	1000 ml

Solution B:

KH_2PO_4	3.4 g
ddH_2O up to	500 ml

Adjust solution A to pH 7.6 by titration with solution B.

Phosphate buffered saline (PBS)

A solution of 0.1M PBS, pH 7.2, was used as a general diluent for virus, immunochemicals, and cell line preparations. This solution was prepared as follows:

Solution A:

Na_2HPO_4 $7H_2O$	26.8 g
NaCl	8.5 g
ddH_2O up to	1000 ml

Solution B:

NaH_2PO_4 H_2O	6.9 g
NaCl	4.3 g
ddH_2O up to	500 ml

Adjust solution A to pH 7.2 by titration with solution B.

Virus propagation

MDMV-A (8, 9) and MDMV-B (ATCC-PV53; 54) were propagated and purified from Sorghum sudanese Piper (Stapf), cv. Trudan 5 (Northrup King Corp., Minneapolis, MN) and Zea mays L. cv. Golden Bantam, respectively. Inoculation of propagation hosts was performed by grinding approximately 0.5 g of infected, symptomatic, tissue in a mortar and pestle (Coors Brewery, Boulder, CO) with 10-15 ml of phosphate buffer. Carborundom (600 mesh)-dusted leaves were mechanically inoculated with infected sap by gently rubbing with the thumb and forefinger dipped in

inoculum. To compensate for increased transpiration through wounds, plants were watered immediately following inoculation.

Purification of MDMV

Concentration and purification of virus was performed by a modification of the procedure of Langenberg (82). All centrifugations were performed at 4 C with rotors cooled to the same temperature. A typical purification consisted of 1500 g of infected tissue. The reagents used for virus purification were prepared as described below.

Grinding buffer (0.1M ammonium citrate, pH 6.0, containing 1.0% polyvinylpyrrolidone and 0.5% 2-mercaptoethanol) Infected plant tissue was triturated in this buffer during the initial purification steps. Addition of 2-mercaptoethanol inhibits oxidation and subsequent activation of host viral inhibitors. This buffer was prepared as follows:

$(NH_4)_2HC_6H_5O_7$	22.62 g
ddH_2O up to	1 liter

Adjust the pH to 6.0 with solid KOH, then add:

polyvinylpyrrolidone (Sigma #PVP-40)	10.0 g
2-mercaptoethanol (Sigma #M-6250)	5.0 ml

Extraction buffer (0.1M ammonium citrate, pH 6.0, containing 0.1M 2-mercaptoethanol) This buffer was used to resuspend the polyethylene glycol (PEG) precipitated virus and prepare the sucrose cushion. The buffer consisted of:

$(NH_4)_2HC_6H_5O_7$	22.62 g
ddH_2O up to	1 liter

Adjust the pH to 6.0 with solid KOH, then add:

2-mercaptoethanol	7.0 ml

Suspension buffer (0.1M ammonium citrate, pH 6.0) This buffer was used in the final purification steps and is the purified virus storage buffer. The buffer consisted of:

$(NH_4)_2HC_6H_5O_7$	22.62 g
ddH_2O up to	1 liter

The pH was adjusted to 6.0 with solid KOH.

Purification procedure Young, symptomatic leaves on the propagation host were harvested approximately 10-14 days following inoculation. Infected tissue was triturated in a commercial sized Waring blender with an equal volume (w/v) of grinding buffer. Ice was added occasionally to reduce the temperature of the grinding mixture. Following maceration, the sap was expressed through two layers of cheesecloth. Foam, which may be

present on the collected sap, was allowed to settle for 20-30 min. The crude sap was blended with carbon tetrachloride to a concentration of 5 % (v/v) for 10 sec and was immediately centrifuged at 6000 x g for 10 min. This process clarifies the crude sap, as carbon tetrachloride denatures and precipitates a variety of plant cell constituents. Following centrifugation, the supernatant was filtered through glass wool to eliminate residual carbontetrachloride.

Virus was precipitated from the clarified sap by addition of 0.25% (v/v) Triton X-100 (Sigma #T-6878) and 6.0% (w/v) polyethylene glycol (PEG), molecular weight = 6000. The mixture was stirred for 30 min at room temperature and then centrifuged at 9200 x g for 20 min. The supernatant was discarded and the pellets, containing virus, were resuspended in extraction buffer and centrifuged at 8000 x g for 10 min. The supernatant was collected and the pellets were extracted 2-3 times with extraction buffer. The initial supernatant and each extraction supernatant were combined.

Virus was further separated from plant constituents by centrifugation at 68,000 x g for 1.5 hr through 8.0 ml of 20% sucrose (w/v; Sigma #S-9378) in 25 x 89 Ultra Clear centrifuge tubes (Beckman Instruments Inc., #344058). The supernatant, containing plant contaminants, was discarded

and the viral pellets were resuspended overnight in suspension buffer. Remaining plant constituents were removed by low-speed centrifugation at 6000 x g for 20 min. The supernatant was collected and the pellets were extracted once or twice with suspension buffer. The initial supernatant and each extraction supernatant were combined.

Virus was further purified through linear 10-40 % sucrose gradients in an SW 27 swinging bucket rotor (Beckman) centrifuged at 22,000 x g for 2 hr. The gradients were fractionated from the bottom using an ISCO Density Gradient Fractionator model 640. Virus containing zones were detected spectrophotometrically at A_{254} and collected. Pellets obtained in the centrifuge tubes, as a result of virus aggregation, were resuspended in suspension buffer and centrifuged through another sucrose gradient.

Sucrose in the collected virus peaks was removed by overnight dialysis against suspension buffer. The dialysate was concentrated by ultracentrifugation for 2 hr at 65,000 x g. The purified virus was resuspended in 2-4 ml of suspension buffer and concentrations were estimated spectrophotometrically by using $A^{0.1\%}_{260} = 2.4$ (112).

Glassware and plasticware preparation

Before use, all glassware and plasticware (except that which was used for virus purification) was washed three times with distilled water and then rinsed briefly with 10% hydrochloric acid in ddH_2O. This was followed by three more rinses with distilled water and one final rinse with ddH_2O. Use of this procedure was most critical when preparing tissue culture media and reagents.

Production of Monoclonal Antibody-Secreting Hybridomas

The technique used for the production of monoclonal antibodies is a modification of the procedure described by Van Deusen and Whetstone (149). Significant modifications including duration of PEG exposure and pH of fusion media, have recently been reported by Lane (81) and Westerwoudt (157), respectively. The specialized reagents and media are described below.

Dulbecco's modified eagle medium (DMEM)

Dulbecco's modified eagle medium was used as the basic nutritive medium. Modifications of this medium were prepared for specialized uses. The basic nutritive medium consisted of:

DMEM high glucose (Gibco

Laboratories, Grand Island, NY; #430-2100)	1 package (10 g)
$NaHCO_3$	15.0 g
HEPES (Sigma #H-3375)	29.9 g
ddH_2O up to	5 liters

The pH was adjusted to 7.2-7.4 with NaOH saturated ddH_2O and filter sterilized through a sterile 0.22 um x 90 mm Zetapore Membrane Filter (AMF Cuno, Meriden, CT; #NM090-01-020SP). The medium was stored in 500 ml volumes at 4 C. Serum added to the medium was a mixture of 2 parts horse serum (Gibco #230-6050) and 1 part calf serum (Gibco #230-6170). Two bottles of DMEM containing 10% and 20% serum were used to prepare specialized media described below. All specialized media was stored at 4 C unless stated otherwise.

100X L-glutamine

A 100X solution of L-glutamine was prepared by dissolving 2.92 g of L-glutamine (Sigma #G-3126) into 100 ml of ddH_2O. The L-glutamine solution was filter sterilized through a sterile 0.2 um syringe filter (Schleicher and Schull, Dessel, W. Germany; #462-200) and stored at -20 C in 1.0 ml aliquots. L-glutamine was a required nutrient used in the preparation of all cell growth media.

Regular growth medium (DMEM-R)

DMEM-R, used for the growth of all established cell lines, was prepared by adding 1.0 ml of 100X L-glutamine to 100 ml of DMEM containing 10% serum. DMEM-R not used within two weeks was discarded.

8-azaguanine medium (DMEM-8-aza)

8-azaguanine (8-aza) medium was used to eliminate Sp2/0 mutants resistant to aminopterin (149). The 8-aza solution was prepared by placing 2.0 mg of 8-aza (Sigma #A-8526) into 1 liter of ddH_2O. The 8-aza was disolved on a magnetic stirrer at 37 C overnight. DMEM-8-aza was prepared as described for DMEM; however, the 8-aza solution was used in place of ddH_2O and the other reagents were scaled down to 1 liter. DMEM-8-aza was used to continuously culture Sp2/0 cells, except 1 week after thawing and 10 days prior to performing cell fusions.

Conditioned medium (CM)

DMEM-R, which has turned orange-yellow due to Sp2/0 growth, is termed conditioned medium. CM is thought to contain certain growth factors which alleviate the need for growth supplements or feeder cell-layers (136). After 2-3 days growth in DMEM-R, Sp2/0 cells are centrifuged in a clinical centrifuge (International Clinical Centrifuges,

Boston, MA) at 225 x g for 10 min and discarded. If not used immediately for media preparations, the supernatant (CM) was stored at -20 C in 100 ml volumes.

Cell viability stain

Trypan blue (Allied Chemical, New York, NY; #508) was used as a cell viability stain when performing all cell counts. The stain was prepared just before use by diluting a stock solution of 4.0 % (w/v) trypan blue in PBS 1:50 with PBS. The membranes of dead cells were permeable to trypan blue and thus stained dark blue. Viable cells remained colorless.

Polyethylene glycol 1000 (PEG-1000)

Polyethylene glycol (molecular weight = 1000 daltons; Hazelton #59-90739) solution was prepared at least 2 hr prior to performing a fusion by solubilizing 2.25 g of PEG-1000 into 2.7 ml of PBS at 37 C. PEG-1000 was utilized as the chemical fusogen during cell fusion procedures.

Fusion medium (DMEM-F)

Cell fusion medium was used to dilute fused cells after incubation with the fusogen PEG-1000. DMEM-F was prepared by adjusting the pH of serum-free DMEM to 8.0

with 1.0N NaOH in ddH_2O. The medium was filter sterilized through a 0.2 um syringe filter and stored in 25 ml volumes. Use of this high pH fusion medium significantly increases the number of successful hybridomas produced during the fusion procedure (157).

Hypoxanthine-aminopterin-thymidine medium (DMEM-HAT)

Hypoxanthine-aminopterin-thymidine (HAT) medium was used to select successful mouse spleen and Sp2/0 cell fusions. Sp2/0 cells lack the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) gene and are thus susceptable to aminopterin. In successful mouse spleen and Sp2/0 cell fusions (hybridomas), the HGPRT gene is supplied by the spleen cell. Therefore, the hybridomas can ultilize supplied hypoxanthine and thymidine in an alternate, salvage-pathway for nucleotide synthesis (36). DMEM-HAT was prepared by adding 1.0 ml of sterile 100X HAT solution (Hazelton-Dutchland Inc., Denver, CO; #59-77076) to 100 ml of filtered DMEM-CT. The 100X HAT solution was stored at -20 C in 1.0 ml volumes.

Hypoxanthine-thymidine medium (DMEM-HT)

Hypoxanthine-thymidine (HT) medium was used to wean the hybridomas off HAT medium. The presence of exogenous hypoxanthine and thymidine is neccessary for hybridoma

growth until aminopterin is diluted to tolerable levels by successive feedings or cloning (36). DMEM-HT and 100X HT solution (Hazelton #59-57076) were prepared and stored as described for DMEM-HAT using 100X HT solution in place of 100X HAT solution.

Cloning and thawing medium (DMEM-CT)

Cloning and thawing medium was used in both cell cloning and thawing procedures. DMEM-CT was prepared by adding 50 ml of CM to 50 ml of DMEM containing 20% serum, and adding 1.0 ml of 100X L-glutamine. The medium was filter sterilized through a 0.2 um syringe filter prior to use. DMEM-CT ultilizes the additional growth factors supplied by CM to promote cell growth under stressful conditions.

Cell freezing medium

This medium was used to freeze all valuable cell lines. Cell freezing medium was prepared by adding 10 ml of Dimethyl Sulfoxide (DMSO; Sigma #D-2650) to 90 ml of serum.

Growth and maintenance of the Sp2/0 cell line

The non-immunoglobulin producing myeloma cell line Sp2/0-Ag 14 (Sp2/0) was first generated by Schulman et al.

(127). The Sp2/0 cell line utilized in these experiments was generously donated by R.A. Van Deusen (National Veterinary Services Laboritories, Ames, IA). The cells were grown in stationary suspension in Corning 25 cm^2 tissue culture flasks (T-flasks; Allied Fisher Scientific, Chicago, IL; #10-126-30) with loosened caps kept at 37 C with humidified 5.0 % CO_2 in air. The cells were maintained in continuous culture by passing at a 1:20 dilution in nutritive medium every 3 days. DMEM-8-aza was utilized as the nutritive medium with the following exceptions: 1) cells were fed continuously with DMEM-R 10 days prior to performing a fusion, 2) DMEM-R was used when generating CM, and 3) DMEM-CT was utilized during cell thawing procedures.

Maintenance of healthy, exponentially dividing, Sp2/0 cells free of mutations was essential for the generation of stable hybridomas. To reduce the possibility of cell line mutations, as a result of long-term continuous culture, the following modification of the procedure of Reading (115) was used. Sp2/0 cells were dispensed into several cryotubes and frozen at -100 C as described on pp. 39-40. After 30 days of continuous culture, all Sp2/0 cell cultures were discarded and a fresh culture was established from frozen stocks. This cycle was repeated every 30 days and frozen stocks were replaced every 6

months by freezing cells from a recently thawed and expanded culture.

Immunization of mice with MDMV

All immunizations were performed on female mice of the Balb/C strain (Jackson Labs, Bar Harbor, ME). Purified MDMV was diluted to 50 ug in 0.1 ml of PBS (final concentration 1.0 mg/ml). The diluted virus was mixed 1:1 (v/v) with Freunds' complete adjuvant (Sigma #F-5881) and emulsified by repeated passage of the mixture through an 18 gauge needle. The entire volume was injected into a single mouse by the intraperitoneal route (IP). Five to eight weeks later the mice were given a second IP injection with 25 ug of virus emulsified in Freunds' complete adjuvant as described. Approximately 2 weeks later, the mice were hyperimmunized by intravenous administration of 25 ug of virus in PBS. The mice were sacrificed three days following hyperimunization.

Cell fusion protocol

Hyperimmunized mice were exsanguinated via cardiac puncture and the serum was collected by centrifugation of the clotted blood using a Sure-sep II (General Diagnostics Division of Warmer-Lambert Co., Morris Plains, NJ; #33709). The sera was stored at -20 C and later titered

by ELISA. The sacrificed mouse was soaked in a 400 ml beaker with 300 ml of 70% ethanol (ETOH) in ddH_2O and positioned on the sterile surface of a Biogard laminar flow hood (Baker Co. Inc., Biddeford, MA) with the right flank down. The spleen was aseptically removed and placed in a Corning 60 x 15 mm tissue culture dish (Fisher #08-772-21) with 2.0 ml of DMEM. The spleen was perfused by injection with 10.0 ml of DMEM using a sterile 10 ml syringe barrel (Becton-Dickinson Division, Rutherford, NJ; #5604) equipped with a sterile 26 gauge needle (B-D #5625). The spleen was discarded and the spleen cell suspension was placed into a Corning 50 ml polypropylene centrifuge tube (Fisher #05-538-55). Cell aggregates were disrupted by passing the suspension through a 10 ml pipet several times. An estimate of viable spleen cells was obtained by diluting 0.1 ml of spleen cell suspension 1:50 in cell viability stain. Viable cells were counted in a hemacytometer (AO Instrument Co., Buffalo, NY) and cell numbers were calculated: total cells counted X 1.0 x 10^5 X volume of cell suspension. Generally, the suspension contained 7.0-8.0 x 10^7 viable spleen cells.

Viable Sp2/0 cells were added to the spleen cell suspension at a ratio of 1:2 (Sp2/0:spleen cells) and the cells were centrifuged in a clinical centrifuge at 225 x g for 10 min. The supernatant was discarded and the cell

pellet was immediately resuspended by gently swirling the centrifuge tube.

Note: The following fusion procedure involves use of the highly toxic PEG-1000 as a fusogen; therefore, careful timing of each step was critical.

Over a 15 sec period, 1.0 ml of solubilized PEG-1000 sterilized through a 0.2 um syringe filter was slowly added to the cell suspension. Two ml of DMEM-F was added immediately over a period of 30 sec and the fusion mixture was swirled gently. At this point, 10.0 ml of DMEM-F was added over a 60 sec period, the tube was swirled gently, and the mixture was incubated at 37 C for 5 min.

Following incubation the cells were centrifuged in a clinical centrifuge at 225 x g for 10 min. The supernatant was discarded and the cells were gently resuspended in 1.0 ml of DMEM-HAT. Once fully resuspended, the suspension was diluted with DMEM-HAT to 5.0×10^5 Sp2/0 cells/ml. Using a sawed-off 10 ml pipet (sawed-off pipets were prepared by scoring and snapping off the tip of a long-nose glass 10 ml pipet and fire-polishing the end), the cell suspension was dispensed at 0.2 ml/well in Corning 96-well tissue culture plates (Fisher #08-757-155). The cell cultures were incubated at 37 C with humidified 5.0 % CO_2 in air.

Growth and maintenance of hybridomas

The culture plates were examined daily for media color changes due to contamination. On the 3rd and 7th days post-fusion, the cells were fed by aspirating the spent medium and adding 0.2-0.3 ml/well of DMEM-HAT. After 3 days, hybridomas were observed with a Nikon inverted microscope (Frank E. Fryer Co. Inc., Carpentersville, IL; model 64453); they appeared as refractive colonies of up to 20 cells. An average of 8 hybridoma colonies/well were detected in each of the approximate 600 wells cultured.

Hybridomas were assayed for specific antibody production by indirect ELISA no later than 10 days post-fusion; all positive hybridomas were cloned immediately. Westerwoudt et al. (158) found that on day 14 post-fusion only 35 %, of the original positives assayed on day 10, were still positive. Therefore, it is critical to assay and clone positive hybridomas before day 14 post-fusion. The process of weaning the hybridomas off HAT was performed during the cloning and expansion procedures.

Hybridoma cloning and expansion

The cloning procedure utilized is a modification of the limiting-dilution procedure presented by Galfre and Milstein (36). Significant modifications which alleviate

the need for time-consuming scale-up procedures were developed in our laboratory by R. L. Mernaugh, F. E. Jones, and C. W. Kaspar.

Specific antibody-producing hybridomas were thoroughly resuspended by mixing the contents of a single well with a Falcon cotton-plugged 2.0 ml serological pipete (B-D #7507) equipped with a rubber propipette. One drop (approximately 50 ul) of the cell suspension was transferred to a sterile Falcon 13 X 100 mm tube (B-D #2027) containing 5.0 ml of DMEM-HT. The remainder of the suspension (approximately 0.2 ml) was transferred to a single well of a Corning 24-well tissue culture plate (Fisher #08-757-156) containing 1.0 ml of DMEM-HT. The tube was inverted several times and 1 drop of cell suspension was transferred to a second tube containing 5.0 ml of DMEM-HT. The second tube was inverted several times and the contents of this tube were dispensed at 0.1 ml/well into 48 wells of a 96-well tissue culture plate. The plates were incubated at 37 C with humidified 5.0 % CO_2 in air. Three to four days later, the 96-well tissue culture plates were examined for wells containing single colonies using a low-power (40X) inverted microscope. Cells in 24-well tissue culture plates were fed by aspirating the spent media and adding 1.5 ml of DMEM-HT. When the cells had grown to confluency on the bottom of the well (ap-

proximately 3 days), the contents of that well were frozen.

After 7 days, 0.1 ml of DMEM-CT was added to each well of the 96-well tissue culture plate. Three days later, wells containing single colonies were assayed for specific antibody production by indirect ELISA. An antibody-producing colony from a single well was thoroughly resuspended, transferred, and cloned in DMEM-CT as described previously. The cloning cycle was repeated until all wells containing single colonies were positive for specific antibody production. If no positive colonies were obtained after the first cloning cycle, the hybridomas' frozen counterpart was thawed and cloned in DMEM-CT. Completely cloned cell lines, producing specific McAb, were immediately expanded and frozen.

Cell lines were expanded as follows. A McAb-producing colony from a single well was thoroughly resuspended and transferred to a single well of a 24-well tissue culture plate with 1.0 ml of DMEM-R. Three days later, the cells were fed by aspirating off the spent media and replacing with 2.0 ml of DMEM-R. Once the cells had grown to confluency on the bottom of the well, they were resuspended and the entire volume was transferred to a 25 cm^2 T-flask with 8.0 ml of DMEM-R. The cells were grown to a density of approximately 6.0 x 10^5 cells/ml and

then frozen. These cell lines were also maintained in continuous culture by passing them at a 1:5 dilution in DMEM-R and injected into mice for ascites fluid production.

Cell line freezing procedure

Sp2/0 cells, hybridomas, and McAb-producing cell lines were frozen at -100 C for short-term storage (up to 6 months) or in liquid nitrogen (-196 C) for long term storage (6 months to 1 yr). McAb-producing cell lines were frozen immediately following the final cloning cycle, as continuous culture for more than 14 days may result in loss of antibody production.

Cells to be frozen were cultured in 25 cm^2 T-flasks with 10.0 ml of DMEM-R. When the cell density was approximately 6.0 x 10^5 cells/ml (media color orange-yellow), the cells were centrifuged in a clinical centrifuge at 225 x g for 10 min. The supernatant, which contains a low level (10-50 ug/ml) of specific McAb, was decanted and stored at -20 C in a Corning sterile 50 ml polypropylene centrifuge tube. The pellet was gently resuspended in 5.0 ml of cell frezing medium and dispensed into 5 Nunc 1.2 ml cryotubes (Vangard International, Neptune, NJ; #366656) at approximately 1.2 x 10^6 cells/tube.

Cells cultured in 24-well tissue culture plates were also frozen to eliminate time-consuming cell expansion procedures. When the cells had grown to confluency on the bottom of the well, the entire volume of spent media was aspirated. The cells were resuspended in 1.5 ml of cell freezing medium and transferred to a 1.2 ml cryotube.

The cryotubes were placed into Nunc transport containers (Vangard #241) and packaged in a 20 X 20 X 6 cm styrofoam freezing chamber such that temperature reduction approximates 1 C/min. The freezing chamber was placed at -100 C in a Queue Cryostar cryogenic freezer (Lab Research Products, Lincoln, NE) overnight. The following day the cryotubes were removed from the freezing chamber and either placed directly into liquid nitrogen or placed back at -100 C.

Cell line thawing procedure

The following procedure was used to establish a culture from frozen stocks. The cryotube, containing the culture of interest, was removed from the freezer or liquid nitrogen and placed immediately into a 37 C incubator to thaw. Once thawed, the cryotube was inverted several times to fully resuspend the cells. The cell suspension was transferred to a Corning sterile 15 ml polypropylene centrifuge tube (Fisher #05-538-53D) with

10.0 ml of DMEM and centrifuged at 225 x g for 10 min. The supernatant was discarded and the pellet was gently resuspended in 2.0 ml of DMEM-CT. The cell suspension was dispensed at 1.0 ml/well into 2 wells of a 24-well tissue culture plate and incubated at 37 C with humidified 5.0 % CO_2 in air.

Three days later the cells were fed by aspirating the spent media and replacing with 2.0 ml of DMEM-CT. When the cells had grown to confluency on the bottom of the well they were transferred to 25 cm^2 T-flasks and fed with DMEM-R as described.

Ascitic fluid production

Hybridoma cells injected into compatible mice induce formation of ascites tumours and production of ascites fluid containing high levels (up to 10 mg/ml) of specific antibody. The parameters affecting ascitic fluid production have been optimized (136).

Specific McAb-producing hybridomas were maintained in continuous culture as described. When the cells grew to an approximate density of 6 x 10^5 cells/ml (one T-flask with 10 ml of cells was sufficient to inject 3 mice), they were collected by centrifugation at 225 x g for 10 min. The supernatant was decanted and stored at -20 C as described. The hybridoma pellet was gently resuspended

and washed twice with PBS warmed to 37 C. The final pellet was resuspended in sufficient PBS to give 1.0 x 10^7 cells/ml. Pristane-primed male mice of the Balb/C strain (mice were pristane-primed by injection, IP, with 0.5 ml of 2, 6, 10, 14, tetramethylpentadecane (pristane; Sigma #T-7640) two weeks previous) were injected, IP, with 0.2 ml of hybridoma suspension (approximately 2.0 x 10^6 cells/mouse).

The mice were observed daily for abdominal swelling. When swelling was pronounced (7-10 days), the tumour was drained by inserting a sterile 18 gauge, 3.8 cm needle (B-D #5196) into the peritoneal cavity and allowing the ascitic fluid to drain into a Corning sterile 50 ml polypropylene centrifuge tube. The mice were usually tapped 2-3 days later and then sacrificed. The ascitic fluid was centrifuged at 1000 x g for 10 min to remove cells. The supernatant was assayed for specific McAb by indirect ELISA and immediately stored at -20 C in 5.0 ml aliquots. Using this procedure, each mouse produces 10-20 ml of ascitic fluid and usually survives 14-16 days after injection with hybridomas.

Indirect enzyme-linked immunosorbant assay (ELISA) for the detection of MDMV specific antibody

A successful fusion usually results in the culture of 500-600 wells containing hybridomas. The loss or inactivation of antibody producing chromosomes frequently yields both non-producing and producing hybridomas in the same well (158). Goding (42) has shown that antibody production may take up to 30-50 % of the cellular energy; therefore, hybridomas not producing antibody may divide more rapidly and overgrow the useful hybridomas. The loss of chromosomes and the danger of overgrowth decreases with time (158). Thus a simple, rapid, and sensitive assay must be incorporated to detect desirable hybridomas and allow cloning shortly after fusion.

Modifications of the indirect ELISA described by Voller et al. (156) are utilized for the rapid, routine, detection of specific antibody. This ELISA was designed such that positive results could be easily visualized or quantitated spectrophotometrically. The buffers and reagents used were prepared as follows:

BLOTTO (5.0 % non-fat dry milk in PBS)

BLOTTO was used to block any protein binding sites remaining after coating microtitration plates with antigen. BLOTTO is very inexpensive to prepare, but cannot be

utilized if a peroxidase enzyme system is incorporated as non-fat dry milk contains exogenous peroxidase activity. BLOTTO consisted of:

Non-fat dry milk (Hy-Vee Food Stores, Ames, IA)	10.0 g
Antifoam A emulsion (Sigma #A-5758)	70 ul
0.1M PBS, pH 7.2 up to	200 ml

BLOTTO was prepared fresh daily and 200 ml was sufficient to block 3 96-well microtitration plates.

Wash buffer (0.01M PBS, pH 7.4, containing 0.05 % Tween-20) Wash buffer was used to remove unbound reactants and reduce non-specific reactions. This buffer was prepared fresh daily and prepared as a 5X concentrate if an assay required use of more than three microtitration plates. The wash buffer and 5X concentrate were prepared as follows:

	1X	5X
NaCl	42.5 g	212.5 g
KH_2PO_4	1.0 g	5.0 g
KCl	1.0 g	5.0 g
Na_2HPO_4	5.8 g	29.0 g
Tween-20 (Sigma #P-1379)	2.5 ml	12.5 ml
ddH_2O up to	5 liters	5 liters

Substrate buffer (10.0 % diethanolamine in ddH_2O, pH 9.8) Substrate buffer was used to dilute the alkaline phosphatase substrate, p-nitrophenyl phosphate (p-NPP). The p-NPP was obtained in tablet form (5 mg/tablet; Sigma #104-105) and diluted to 1.0 mg/ml in 5.0 ml of substrate buffer immediately before use. Conveniently, one p-NPP tablet provided sufficient substrate for one 96-well microtitration plate. The substrate buffer was stored at 4 C and the p-NPP tablets were stored at -20 C until use. Substrate buffer was prepared as follows:

Diethanolamine (Sigma #D-8885)	50.0 ml
$MgCl\ 6H_2O$	50.0 mg
ddH_2O up to	500 ml

Stop solution (3N NaOH) To quantitate results spectrophotometrically it was neccessary to terminate the enzyme-substrate reaction. This was accomplished by adding 50 ul of 3N NaOH/well.

Indirect ELISA procedure Unless stated otherwise, each step of the ELISA was incubated in a humidified chamber for 1 hr at room temperature or overnight at 4 C.

Wells of a Nunc certified Immuno I 96-well microtitration plate (Vangard #4-39454) were coated with 50 ul of purified MDMV/well diluted to 5.0 ug/ml in 5.0 ml of PBS. Following incubation, the plate was washed by

first discarding the MDMV solution and then submerging the plate into a 2 liter beaker filled with wash buffer. When all the wells had filled, the plate was removed and the wash buffer was sharply "flicked" from the wells of the plate. This was repeated 4 times in rapid succession and the plate was incubated for 5 min with wash buffer following the last cycle. After the final wash cycle, the entire contents of the beaker was discarded and replaced with fresh wash buffer. The plate was tapped dry on paper towels and unbound protein sites were blocked by completely filling all the wells with BLOTTO. The plate was incubated and washed as described. The plate could then be used immediately or stored at 4 C up to 4 days without loss of specificity or sensitivity.

Specific antibody was bound to immobilized MDMV by adding (50 ul/well) hybridoma culture media which had supported cell growth for 3 or more days (media color orange-yellow). Typical controls added to each plate included hyperimmune mouse serum diluted 1:1000 in PBS (positive control), normal mouse serum diluted 1:1000 in PBS, and CM (negative controls). The plate was incubated and washed 3 times as described. Hybridomas were assayed for the production of antibody classes immunoglobulin G (IgG) and immunoglobulin M (IgM). This was performed by adding (50 ul/well), a mixture of alkaline phosphatase

conjugated rabbit anti-mouse IgG (Sigma #A-1902) and alkaline phosphatase conjugated goat anti-mouse IgM (Sigma #A-7784), both diluted 1:1000 in wash buffer with 1.0 % fraction V bovine serum albumin (BSA; Sigma #A-4503). The plate was incubated and washed 3 times as described. Then, 50 ul of diluted substrate was added to each well and color development was allowed to procede for 30-60 min before the addition of stop solution.

For quantitation experiments, A_{410} of each well was measured in a Dynatech Minireader II (Dynatech Laboratories Inc., Alexandria, VA; #011-930-0500). The color development of each well could also be determined visually. Positive wells were distinguished as various intensities of yellow when compared to colorless negative controls. Positive hybridomas were cloned and expanded as described.

Immunoglobulin class and subclass determination

For protein A purification purposes, it was necessary to determine the Ig class and subclass secreted by cloned cell lines. The IgM class cannot be purified using protein A chromatography and purification of the IgG class expresses subclass varability (43).

The McAb Ig class was determined by a modification of the indirect ELISA procedure described previously. Each

cloned McAb producing cell line was represented by two wells on a microtitration plate previously coated with homologous virus and blocked with BLOTTO. To one of these wells was added 50 ul of alkaline phosphatase conjugated rabbit anti-mouse IgG diluted 1:1000 in wash buffer containing 1.0 % BSA. The same dilution of alkaline phosphatase conjugated goat anti-mouse IgM was added to the other well. The McAb class was determined by observing which Ig class specific conjugate reacted with the McAb following addition of substrate.

Ig subclass determination was only performed with IgG secreting cell lines. All neccessary immunochemicals and reagents were purchased as a MonoAb-ID EIA Kit (Zymed Laboratories Inc., San Francisco, CA; #90-6550). The procedure was performed in a Nunc 96-well microtitration plate as described by the subclass kit manufacturer. Positive wells were blue-green when compared to the colorless negative controls. The McAb subclass was determined by observing which IgG subclass specific rabbit anti-mouse reacted with the McAb following addition of substrate.

On occasion the substrate solution supplied with the kit was inactive and was thus replaced by the following peroxidase substrate solution.

ABTS substrate (2, 2, azino-di-[3-ethylbenzthiazoline sulfonic acid])

Solution A (0.05M citric acid, pH 4.0):

$HOCCOOH(CH_2COOH)_2\ H_2O$	1.05 g
ddH_2O up to	100 ml

Adjust the pH to 4.0 with 1.0N NaOH.

Solution B (40 mM ABTS):

ABTS (Sigma #A-1888)	45 mg
solution A	2.0 ml

Substrate mixture:

solution A	5.0 ml
solution B	20 ul
3.0 % H_2O_2 in ddH_2O	25 ul

Each solution was stored at 4 C in separate brown bottles and the substrate mixture was prepared fresh daily. Old solutions react non-specifically with proteins and were thus replaced every 2 months.

Polyacrylamide Gel Electrophoresis (PAGE) of Viral Polypeptides

Preparation of viral proteins for SDS-PAGE

To obtain several protein polypeptides of various molecular weights, the viral coat protein was denatured

and proteolytically digested. Initially, purified virus was treated with an excess of 2-mercaptoethanol and SDS. The 2-mercaptoethanol reduces disulfide bonds and the SDS binds to regions of the protein to cause disruption of intramolecular protein associations. The denatured protein subunits were then proteolytically cleaved into fragments of various molecular weights. The amount of proteolytic enzyme and the digestion period were optimized to produce a wide range of fragment mobilities during SDS-PAGE. The buffers used for this procedure are described below.

Dialysis buffer (0.125M Tris-HCl, pH 7.6, containing 0.1% 2-mercaptoethanol) This reducing buffer was used to dialyze purified virus preparations prior to SDS degradation. Dialysis buffer consisted of:

Trizma HCl (Sigma #T-3253)	60.6 g
Trizma base (Sigma #T1503) or Tham	13.9 g
2-mercaptoethanol	4.0 ml
ddH_2O up to	4 liters

If neccessary, adjust pH to 7.6 with 1.0N NaOH or 1.0N HCl.

Degradation buffer (0.125M Tris-HCl, pH 6.8, containing 1.0% SDS and 1.0% 2-mercaptoethanol) This buffer was used to degrade and solubilize viral proteins prior to proteolytic digestion. Degradation buffer consisted of:

Trizma HCl	1.76 g
Trizma base or Tham	0.168 g
Laural sulfate (SDS; Sigma #L-5750)	1.0 g
2-mercaptoethanol	1.0 g
ddH_2O up to	100 ml

Adjust pH to 6.8 with 1.0N HCl.

Sample buffer In addition to bromophenol blue used as a tracking dye, sample buffer also contained an excess of 2-mercaptoethanol and SDS to maintain the denatured state of the disrupted protein fragments. This buffer was prepared as follows:

Tris base (Bio-Rad #161-0716)	0.76 g
SDS (Bio-Rad #161-0301)	1.0 g
2-mercaptoethanol	2.0 ml
Glycerol	15.0 ml
Bromophenol blue	1 mg
ddH_2O up to	100 ml

Adjust the pH to 6.8 with 1.0N HCl and store at room temperature.

Procedure for viral protein preparation Purified MDMV was adjusted to a concentration of approximately 2.0 mg/ml with dialysis buffer and dialyzed overnight at 4 C against two changes of dialysis buffer. The dialyzed virus was adjusted to a concentration of 1.0 mg/ml with dialysis buffer. An equal volume of degradation buffer was added and the viral proteins were degraded by placing the mixture in a boiling water bath for 20 min. Once degraded, the solubilized proteins were immediately cooled on ice and stored at 4 C until use.

The enzyme used for the digestion of both MDMV-A and MDMV-B viral proteins was a protease isolated from Staphylococcus aureus, strain V8 (V8 protease; Sigma #P-8400). During optimization procedures, the lypholyzed V8 protease preparation was reconstituted to 2.0, 1.0, and 0.5 mg/ml in dialysis buffer. To each 100 ul of viral protein to be digested, 20 ul of V8 protease was added. The digestion mixtures were incubated at 37 C for 2, 4, or 6 hr. Following incubation, the digestion mixtures were placed in a boiling water bath for 10 min and then immediately cooled on ice. The protein fragments were analyzed by SDS-PAGE using a Bio-Rad mini-gel as described. Further digestions were performed using the optimized V8 protease concentration and digestion time.

Prior to electrophoresis, digested protein samples

were diluted (usually 1:1) to the desired concentration with sample buffer. To solubilize and maintain the denatured state of the proteins, each diluted sample was placed in a boiling water bath for 2 min and cooled on ice before application to the gel.

Discontinuous SDS-PAGE (disc-SDS-PAGE)

Separation of digested MDMV protein fragments was accomplished using the disc-SDS-PAGE system described by Laemmli (80) with stacking and resolving gels of 4.0 % and 15.0 % acrylamide, respectively. Disc-SDS-PAGE efficiently stacks and separates protein fragments by electrophoresis through use of a high pH glycine electrode buffer and a low pH Tris buffered stacking gel. Briefly, dilute samples are added to the upper electrode buffer chamber. When an electrical potential is applied to the system, current flow induces the flow of glycine ions in the electrode buffer. The protein fragments and the glycine ions migrate into the low pH stacking gel. Titration at low pH produces an increased negative charge on the polypeptides and a reduced negative charge on the glycine ions. As a result, the protein fragments have a greater mobility than the glycine ions and thus accumulate ahead of the glycine front. Furthermore, chloride ions in the Tris buffered stacking gel, carrying a negative

charge, migrate even faster than the protein fragments. This results in the concentration and stacking of different molecular weight proteins perpendicular to the current flow.

Continued current flow and migration into the high pH resolving gel results in a higher glycine ion negative charge and a reduced protein negative charge. Therefore, each, pre-stacked, protein fragment migrates through the resolving gel as a function of its unique charge and shape. The specialized buffers required to perform Disc SDS-PAGE are described below.

Acrylamide solution Acrylamide solution contains the acrylamide monomer and the cross-linker N, N'-methylene-bis-acrylamide (bis-acrylamide). The monomer and cross-linker remain relatively stable when mixed; however, when the free-radical initiator ammonium persulfate (APS) is added along with the catalyst N, N, N', N'-tetramethylethylenediamine (TEMED), polymerization occurs. A 30 % stock acrylamide solution was prepared as follows:

Acrylamide (Bio-Rad #161-0101)	30.0 g
Bis-acrylamide (Bio-Rad #161-0201)	0.8 g
ddH_2O up to	100 ml

Acrylamide solution was stored at 4 C in the dark for a

maximum of 30 days.

Resolving gel buffer (1.5M Tris-HCl, pH 8.8) This high pH buffer was used to prepare the 15.0 % resolving gel. Resolving gel buffer consisted of:

Tris base	18.17 g
Disodium ethylenediamine tetra-acetate (EDTA)	0.15 g
ddH_2O up to	100 ml

Adjust pH to 8.8 with 1.0N HCL and store at 4 C.

Resolving gel preparation (15.0 % acrylamide, 0.375M Tris-HCl, pH 8.8) The resolving gel separates protein fragments which have been concentrated and stacked in the stacking gel. All buffers were warmed to room temperature prior to use. The monomer volume required to pour two mini-gel or two 16 cm gel sandwiches was 10.0 and 60.0 ml, respectively. The resolving gel was prepared as follows:

Total Monomer	10.0 ml	60.0 ml
Resolving gel buffer	2.5 ml	15.0 ml
Acrylamide solution	5.0 ml	30.0 ml
10 % (w/v) SDS in ddH_2O	0.1 ml	0.6 ml
ddH_2O	2.35 ml	14.1 ml

The mixture was degassed for 15 min at room temperature and used immediately.

Stacking gel buffer (0.5M Tris-HCl, pH 6.8) This low pH buffer increases the negative charge on proteins and thus facilitates protein fragment stacking in the 4.0 % gel. Stacking gel buffer consisted of:

Tris base	6.06 g
ddH_2O up to	100 ml

Adjust pH to 6.8 with concentrated HCl and store at 4 C.

Stacking gel preparation (4.0 % acrylamide, 0.125M Tris-HCL, pH 6.8) The combination of low pH and low acrylamide concentration facilitates charge stacking of protein fragments in the stacking gel. All buffers were warmed to room temperature prior to use. The monomer volume required to pour two mini-gel or two 16 cm gel sandwiches was 10.0 and 20.0 ml, respectively. The stacking gel was prepared as follows:

Total Monomer	10.0 ml	20.0 ml
Stacking gel buffer	2.5 ml	5.0 ml
Acrylamide solution	1.3 ml	2.6 ml
10 % (w/v) SDS in ddH_2O	0.1 ml	0.1 ml
ddH_2O	6.1 ml	12.2 ml

The mixture was degassed for 15 min at room temperature and used immediately.

Electrode buffer (0.025M Tris-HCl, pH 8.3) This buffer contains a high concentration of glycine ions to allow current flow from the upper buffer chamber through the gel to the lower buffer chamber. Electrode buffer consisted of:

Tris base	12.1 g
Glycine (Sigma #G-7126)	57.6 g
SDS	4.0 g
ddH_2O up to	4 liters

Adjust pH to 8.3 with concentrated HCl.

Gel fixative and staining solution The addition of methanol to this solution prevents gel shrinkage and thus distortion. In addition, acetic acid fixes the gel during staining procedures. The gel fixative and staining solution consisted of:

Methanol	200.0 ml
Glacial acetic acid	50.0 ml
Brilliant (Coomassie) blue R (Sigma #B-0630)	0.5 g
ddH_2O up to	500 ml

The gel fixative and destaining solution was saved after every use and could be reused several times.

Gel destain solution Gel destain solution consisted of:

Methanol	400.0 ml
Glacial acetic acid	100.0 ml
ddH_2O up to	1 liter

Casting and running miniature Disc SDS-polyacrylamide gel The Bio-Rad Mini-Protean II slab gel system (#165-2900) was used to prepare all miniature gels (mini-gel). The mini-gel (7 cm X 8 cm X 0.75 mm) could be run 5 times faster than conventional 14 cm X 16 cm gels. Thus, the mini-gel was utilized almost exclusively to optimize enzyme concentrations and digest times, and to observe digested preparations before running large samples on the 14 X 16 gel system.

The gel sandwich plates and the electrophoresis chamber were assembled as described by the manufactures' instructions. The resolving gel was poured into one sandwich at a time using 5.0 ml of the degassed resolving gel preparation. The resolving gel monomer was poured into a 10.0 ml syringe barrel equipped with a 25 gauge 1.6 cm needle (B-D #5122) placed into a rubber stopper to prevent leakage. The acrylamide was polymerized by adding 2.5 ul of TEMED (final concentration 0.05 %; Bio-Rad #161-0800) and 25 ul of 10 % APS [final concentration 0.05 %;

10 % APS was prepared fresh daily by dissolving 100 mg of APS (Bio-Rad #161-0700) in 1.0 ml of ddH_2O]. The polymerization mixture was swirled momentarily and quickly poured through the syringe needle into the gel sandwich to a level of 5.0 cm. The resolving gel was immediately overlaid with water-saturated butanol using a pasteur pipete and bulb and allowed to polymerize for 1 hr. Following polymerization, the overlay solution was rinsed off by forcing a gentle stream of ddH_2O through the spout of a wash bottle. The area above the resolving gel was blotted dry using Whatman 3MM filter paper.

As with the resolving gel, only one stacking gel was polymerized at a time. A well-forming comb was placed into the gel sandwich and tilted slightly to prevent air from being trapped under the comb teeth. Five ml of the stacking gel monomer were placed into the 10.0 ml syringe barrel and polymerized by adding 5.0 ul of TEMED (final concentration 0.1 %) and 25 ul of 10 % APS (final concentration 0.05 %). The polymerization mixture was swirled momentarily and added to completely fill the gel sandwich. The comb was properly aligned and the gel was allowed to polymerize without overlay solution for 1 hr. After polymerization, the comb was slowly removed and the wells were rinsed with ddH_2O.

The gels were attached to the inner cooling core as

described by the manufacturer and the buffer chambers were filled with electrode buffer. The samples, diluted in sample buffer, were added (20 ul/well) with a 25 ul Hamilton syringe (Hamilton Co., Reno, Nevada). The syringe barrel was thoroughly rinsed in ddH_2O between samples. The polypeptides were separated by electrophoresis from the anode (+) to the cathode (-) at 200 volts (constant voltage setting) for 45-50 min.

Separated polypeptides were stained by incubating the resolving gel in gel fixative and staining solution overnight. The gel was destained the following day with several changes of gel destaining solution over a 1-2 hr period. At this point, the gel could be stored permanently by drying under vacuum onto Whatman 3MM filter paper.

Casting and running conventional Disc SDS-polyacrylamide gel The Bio-Rad Protean II slab gel system (#165-1802) was used in the preparation of all 14 cm X 16 cm X 1.5 mm slab gels. Because of their size and resolution capabilities these slab gels were utilized to separate polypeptides for subsequent immunoblot analysis.

The gel sandwich plates and the gel chamber were assembled as described by the manufacturer. The resolving gel was polymerized in one gel sandwich at a time using

30.0 ml of the resolving gel preparation. Degassed resolving gel monomer was poured into a 50.0 ml syringe barrel equipped with an 18 gauge 3.8 cm needle placed in a rubber stopper to prevent leakage. The gel was polymerized by adding 15 ul of TEMED (final concentration 0.05 %) and 150 ul of APS (final concentration 0.05 %). The polymerization mixture was swirled momentarily and poured through the syringe needle into the gel sandwich to a level of 11.0 cm. The gel was immediately overlaid with water-saturated butanol and allowed to polymerize for 1 hr. When the gel was completely polymerized, the overlaying solution was rinsed off and the surface of the resolving gel was blotted dry as described.

As with the resolving gel, one stacking gel was polymerized at a time using 10.0 ml of stacking gel preparation. The degassed stacking gel monomer was placed into the 50.0 ml syringe barrel and polymerized by adding 10 ul of TEMED (final concentration 0.1 %) and 50 ul of 10 % APS (final concentration 0.05 %). The polymerization mixture was swirled momentarily and poured through the syringe needle to a level of 2.0 cm. A well-forming comb was not used; rather, the gel was immediately overlaid with water-saturated butanol and allowed to polymerize for 1 hr. After rinsing off the overlay buffer, the gel was used immediately or stored at room temperature until the

following day. If the gel was stored overnight, approximately 10.0 ml of stacking gel buffer, diluted 1:5 in ddH_2O, was placed on top of each gel to prevent dehydration.

A 1.0 ml sample of digested virus was diluted 1:1 with sample buffer and prepared for electrophoresis as described. Both buffer chambers on the electrophoresis cell were filled with electrode buffer and the entire sample was evenly applied to the stacking gel surface using a 10.0 ml syringe barrel equipped with an 18 gauge 3.8 cm needle. The sample became evenly distributed over the entire stacking gel surface within 30 sec. Electrophoresis through the stacking gel was initially at 150 volts (approximately 1 hr) with 2 gels. The power was increased to 250 volts as the sample migrated through the resolving gel. Electrophoresis was terminated when the bromophenol blue tracking dye eluted off the bottom of the resolving gel (total of 5-6 hr). At this point, portions of the resolving gel were fixed and stained as described or the entire resolving gel was transferred to a nitrocellulose membrane for use in immunoblot procedures.

Immunoblot Analysis Of Monoclonal Antibodies

Electrophoretic separation of protein fragments and subsequent analysis of the polypeptides on nitrocellulose blots was utilized to demonstrate epitope specificity of antibody from cloned McAb-producing cell lines. Briefly, viral proteins were enzymatically digested and the polypeptides were separated by discontinuous polyacrylamide gel electrophoresis (PAGE) in the presence of sodium-dodecyl sulfate (SDS) as described by Laemmli (80). The polypeptides were then electrophoretically transferred from the gel electropherogram and non-covalently bound to a nitrocellulose solid-phase as first described by Towbin et al. (147) and recently reviewed by Towbin and Gordon (146). McAbs which specifically bound to the immobilized protein fragments could then be detected using enzyme immunoassay techniques. Differences in protein binding patterns were interpreted as suggesting different epitope specificity exhibited by the McAbs.

Transfer of proteins from gel electropherogram to nitrocellulose membrane ("western blotting")

Electrophoretic elution of polypeptides from a gel electropherogram to a solid-phase nitrocellulose (NC) membrane results in a high resolution copy of the original

gel electropherogram. Individual NC bound polypeptides can be specifically identified using enzyme immunoassay or a non-specific amido black stain can be used for visualization of all polypeptides. The buffers and reagents used during the transfer and total protein staining procedures are described below.

Transfer buffer (0.025M Tris-HCL, pH 8.3) This buffer contains methanol and SDS to facilitate the elution and binding of protein fragments to the NC support. Transfer buffer consists of:

Tris base	12.1 g
Glycine	57.7 g
Methanol	1 liter
ddH_2O up to	4 liters

Adjust the pH to 8.3 with concentrated HCl.

Nitrocellulose staining solution A solution of amido black was used for total protein staining on the NC blot. The staining solution consisted of:

Naphthol blue black (amido black; Sigma #N-3005)	0.1 g
Methanol	45.0 ml
Glacial acetic acid	10.0 ml
ddH_2O up to	100 ml

Nitrocellulose staining solution was saved after every use

and reused several times.

Nitrocellulose destaining solution Nitrocellulose destaining solution consisted of:

Methanol	90.0 ml
Glacial acetic acid	2.0 ml
ddH_2O up to	100 ml

Protein transfer and nitrocellulose staining procedures Electro-elution of protein fragments from the gel electropherogram and subsequent binding to the NC membrane was perfomed in a home-made transfer cell, of a design similar to the commercially available cell from Bio-Rad. Plastic gloves were worn to prevent non-specific protein contamination of the NC membrane during handling.

The transfer assembly was prepared in a baking dish filled to approximately 3.0 cm with transfer buffer. A plastic support was placed in the baking dish and layered with a Scotch-brite pad, 3 sheets of Whatman 3MM filter paper, the resolving gel, and a sheet of type HAHY 0.45 um NC membrane (Millipore #HAHY-000-10). The NC was carefully positioned over the resolving gel and air bubbles were gently removed. The transfer assembly was completed by reversing the stack beginning with the filter paper. If two gels were transferred simultaneously, the stacking procedure was repeated after the last sheet of filter

paper. The transfer assembly was held together with 2 rubber bands, one at each end, and placed into the transfer cell with the nitrocellulose facing the anode (+) and the gel facing the cathode (-).

The cell was filled with transfer buffer and protein transfer was performed at 100 mA (constant mA setting) for 18 hr. When the transfer was complete, excess NC was removed with a razor blade and the gel was stained to confirm that transfer of the protein fragments was complete. The nitrocellulose was cut lengthwise into 1.0 cm wide strips.

During electrophoresis, samples have a propensity to form "smiles" (band patterns curve upward) at both sides of the gel. These smiles are transferred to the NC and thus render the outside NC strips of little use for immunoblot analysis. Therefore, these strips were stained for total protein in nitrocellulose staining solution for 5-10 min and immediately destained for 2-3 min in several changes of nitrocellulose destaining solution. NC membranes are not stable in acidic methanol and should be removed when completely destained. The remainder of the NC strips have identical protein patterns and were utilized for immunoblot analysis.

Immunoblot of nitrocellulose bound polypeptides

The technique of immunoblotting combines the high resolution of PAGE with the sensitivity of enzyme immunoassays. Nitrocellulose serves as the solid phase, such that the immunoblot can be performed as an indirect ELISA with longer incubation and wash steps, and an enzyme-substrate reaction which produces an insoluble product. The buffers and specialized substrate neccessary to perform an immunoblot are described below.

Blocking buffer (0.05M carbonate, pH 9.6, containing 3 % BSA) Blocking buffer was used to quench all unbound sites on the NC membrane. This buffer consisted of:

Na_2CO_3	0.16 g
$NaHCO_3$	0.29 g
BSA	3.0 g
ddH_2O up to	100 ml

If neccessary, adjust the pH to 9.6 with 1.0N NaOH or 1.0N HCl.

Nitrocellulose wash buffer (0.05M Tris-HCL, pH 7.4, containing 0.05 % Tween-20) This buffer was used to remove unbound reactants from the nitrocellulose and reduce background reactions. The nitrocellulose wash buffer consisted of:

Trizma HCl	13.22

Trizma base or Tham	1.94 g
NaCl	17.0 g
Tween-20	1.0 ml
ddH_2O up to	2 liters

If neccessary, adjust the pH to 7.4 with 1.0N NaOH or 1.0N HCl.

AP 7.5 buffer (0.1M Tris-HCl, pH 7.5, containing 0.05 % Triton X-100) This buffer was used to wash the NC membrane and supply neccessary divalent cations for the enzyme-substrate reaction. Wash steps using AP 7.5 buffer are performed quickly to prevent elution of specific proteins by Triton X-100 from the blot. This buffer consisted of:

Trizma HCl	25.4 g
Trizma base or Tham	4.72 g
NaCl	11.7 g
$MgCl_2$ $6H_2O$	0.81 g
Triton X-100 (Sigma #T-6878)	1.0 ml
ddH_2O up to	2 liters

If neccessary, adjust the pH to 7.5 with 1.0N NaOH or 1.0N HCl.

AP 9.5 buffer (0.1M Tris-HCl, pH 9.5) This buffer was used as the final prepatory wash before addition of substrate. AP 9.5 buffer contains divalent cations nec-

cessary for the enzyme-substrate reaction. This buffer consisted of:

Trizma HCl	3.04 g
Trizma base or Tham	21.88 g
NaCl	11.7 g
$MgCl_2$ $6H_2O$	2.03 g
ddH_2O up to	2 liters

Adjust the pH to 9.5 with saturated NaOH.

Nitrocellulose substrate reagent The NC substrate reagent for alkaline phosphatase was prepared as described by Leary et al. (85). The slightly yellow substrate produces a dark purple-blue, insoluble precipitate, which binds to regions of NC where alkaline phosphatase conjugate has been specifically immobilized.

To prepare 15.0 ml of the substrate reagent, 5.0 mg of nitro blue tetrazolium (NBT; Sigma #N-6876) was placed into a 1.5 ml micro-centrifuge tube (USA/Scientific Plastics Inc., Ocala, FL; #USA-505) with 1.5 ml of AP 9.5 buffer and vortexed for 1-2 min. NBT not solubilized was removed by centrifugation for 60 sec in a Beckman microfuge B. The supernatant was decanted into a 50 ml beaker containing 10.0 ml of AP 9.5 buffer warmed to 37 C. The NBT pellet was extracted twice with 1.5 ml of AP 9.5 buffer; each time the supernatant was decanted into

the 50 ml beaker. The centrifuge tube was rinsed with 0.5 ml of AP 9.5 buffer and added to the NBT solution. In a separate micro-cenrifuge tube, 2.5 mg of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma #B-8503) was added to 50 ul of N, N-dimethyl formamide. The BCIP was dissolved by briefly vortexing the solution, which was then added to the NBT solution dropwise with gentle mixing. The NBT-BCIP substrate reagent was prepared fresh daily and stored at 37 C in the dark until use.

Color stop (0.01M Tris-HCl, pH 7.5, containing 1mM EDTA) Color stop was used to terminate color development during the enzyme-substrate reaction. This buffer contains the chelating agent EDTA which sequesters divalent cations neccessary for enzyme function. A 10X color stop concentrate was prepared as follows:

Trizma HCl	1.27 g
Trizma base or Tham	0.24 g
Disodium EDTA	0.37 g
ddH_2O up to	100 ml

If neccessary, adjust the pH to 7.5 with 1.0N NaOH or 1.0N HCl. The 10X concentrate was diluted in ddH_2O prior to use.

Immunoblot procedure The 1.0 cm NC strips, prepared previously, were placed into a baking dish with blocking buffer and incubated at room temperature on a rocker platform for 2-3 hr. The NC membranes were washed on a rocker platform with 3 changes of NC wash buffer over a 30 min period. The NC strips were then placed into separate Seal-a-Meal bags (Dazey Corporation, Industrial Park, KS) with culture media (collected during cell freezing procedures), containing specific McAb, diluted 1:5 in NC wash buffer. One bag contained CM as a negative control. The bags were sealed and incubated overnight as described.

The following day, the NC strips were removed from the bags and washed with NC wash buffer as described. Immobilized McAb was further reacted with a mixture of alkaline phosphatase conjugated rabbit anti-mouse IgG and alkaline phosphatase conjugated goat anti-mouse IgM, both diluted 1:1000 in NC wash buffer with 1.0 % BSA. The strips were incubated in a baking dish for 1 hr and washed as described. After the final wash step, the NC strips were quickly washed 3 times (3-5 min/wash) with AP 7.5 buffer. The strips were then washed with 3 changes of AP 9.5 buffer over a 30 min period. The strips were transferred to a separate baking dish and the NC substrate reagent was added. Color development was allowed to

proceed for 15-20 min before adding the color stop solution. At this point, the NC strips were photographed for a permanent record or stored in the dark with color stop solution.

Isolation of Monoclonal Antibody From Ascitic Fluid

Purification of mouse monoclonal IgM from ascitic fluid

The purification of IgM is a difficult and time-consuming process. The most common method utilized is precipitation of antibody with 40 % saturated ammonium sulfate, followed by gel filtration. However, this procedure may form IgG aggregates which could emerge with IgM during gel filtration (43).

The purification method described below is a modification of the original procedure described by Garvey et al. (39). Briefly, ascitic fluid containing specific IgM was added to a solution of 2.0 % boric acid. The combination of low pH, low ionic strength, and the propensity of boric acid to complex with carbohydrates results in the specific precipitation of IgM. Contaminating low molecular weight proteins are separated from the IgM molecules by sepharose gel filtration. Buffers utilized during the purification of IgM are described below.

2.0 % Boric acid A 2.0 % boric acid solution prepared in ddH_2O was used to specifically precipitate IgM molecules from ascitic fluid. The pH of the solution was approximately 4.0 and was never adjusted. Boric acid solution was prepared immediately before use.

0.1M Tris-HCl saline, pH 8.0 This buffer, used to solubilize the IgM pellet following precipitation with boric acid and as sepharose gel filtration elution buffer, was prepared as follows:

Trizma HCL	35.52 g
Trizma base or Tham	21.2 g
NaCl	34.0 g
ddH_2O up to	4 liters

If neccessary, adjust the pH to 8.0 with 1.0N NaOH or 1.0N HCl.

IgM purification procedure Approximately 10.0 ml of ascitic fluid containing specific IgM was thawed and the lipid layer was removed using a pasteur pipete equipped with a bulb. The ascitic fluid was added drop-wise to 200 ml of boric acid (ascitic fluid:boric acid ratio 1:20) with gentle stirring. The mixture was incubated at room temperature for 30 min and the precipitated antibody was collected by centrifugation at 800 x g for 10 min. The clear supernatant was discarded

resuspended in 1.0 ml of 0.1M Tris-HCl saline, pH 8.0. The solubilized antibody was dialyzed overnight against two changes of 0.1M Tris-HCl saline, pH 8.0, at 4 C. The protein content of the dialysate was estimated using $E^{0.1\%}_{280}$ = 1.4.

Sepharose CL-6B beads (Pharmacia Inc., Pisactaway, NJ; #17-0160-01) were poured into a 58 cm X 1.5 cm i.d. column as described by the manufacturer. Before use, a 10.0 mg/ml solution of Blue Dextran 2000 (molecular weight 2,000 kd; Pharmacia # 17-0360-01) was chromatographed to determine the void volume and visually assess the homogenity of the packed column.

The IgM preparation was diluted 1:1 with glycerol and applied directly to the column bed with a pasteur pipette equipped with a bulb. The sample was chromatographed using 0.1M Tris-HCl saline, pH 8.0, as the elution buffer at a flow rate of 0.25 ml/min. Following elution of the 35.0 ml void volume, 1.0 ml fractions were collected until A_{280} returned to zero. Each fraction was assayed for the presence of specific IgM by indirect ELISA and positive fractions were pooled in 5.0 ml volumes. The protein content was estimated using $E^{0.1\%}_{280}$ = 1.4 and the pooled fractions were stored at -20 C until use.

Purification of mouse monoclonal IgG from ascitic fluid

The purification of mouse monoclonal IgG has been greatly simplified through the use of protein A affinity chromotagraphy. Protein A is isolated from the bacterium Staphlococcus aureus and has a characteristic property of binding IgG with high affinity and specificity. However, one should recognize that there is considerable variation in the binding and stability among the different sub-classes of IgG (43). The buffer used to collect the eluted fractions is described below.

0.1M Tris-HCl, pH 9.0 This buffer was used to increase the pH of fractions containing IgG collected from the protein A column. The Tris buffer was prepared as follows:

Trizma HCl	1.52 g
Trizma base or Tham	10.94 g
ddH_2O up to	1 liter

If neccessary adjust the pH to 9.0 with 1.0N NaOH or 1.0N HCl.

IgG purification procedure Purification of monoclonal IgG was performed with a Bio-Rad Affi-gel protein A MAPS column (#153-6153) using procedures and buffers provided by the manufacture. Briefly, 5.0 ml of

ascitic fluid containing specific IgG was thawed, diluted 1:1 with binding buffer (Bio-Rad #153-6160), and applied directly to the column with a pasteur pipette. Contaminating proteins were eluted from the column with 15 bed volumes of binding buffer. Affinity bound IgG was eluted from the column with 5 bed volumes of a low pH elution buffer (Bio-Rad #153-6160). The eluted IgG was detected spectrophotmetrically at A_{280} and collected in 1.0 ml aliquots into a test tube containing 2.0 ml of 0.1M Tris-HCl, pH 9.0. The final pH of the eluted IgG and Tris solution was approximately 8.0. Each fraction was assayed for the presence of specific IgG by indirect ELISA and positive fractions were pooled in 5.0 ml aliquots. The protein content was estimated using $E^{0.1\%}_{280} = 1.4$ and the pooled fractions were stored at -20 C until use.

Development and Use of a Monoclonal Antibody Based Enzyme-Linked Immunosorbent Assay

ELISA's provide a simple, rapid, and specific method for the detection of viral antigens. Traditionally, polyclonal antisera, derived from rabbits, has been utilized when developing immunological detection assays. However, polyclonal antisera contains a heterogenous population of antibody molecules which may exhibit cross-

reactivity with plant sap or antigens closely related to the immunogen. Although some McAbs may also cross-react with closely related antigens, one can select for specificity.

The ELISA described below was developed based upon the concept of a polyclonal double-sandwich-ELISA (ds-ELISA) as outlined by Clark and Adams (1977). Epitopically distinct hybridomas (determined by immunoblot analysis), secreting antigen specific IgM and secreting antigen specific IgG were utilized as capture and second antibodies, respectively.

Optimization of the double sandwich enzyme-linked immunosorbent assay (ds-ELISA)

The ds-ELISA described below was designed with an IgM capture antibody and an IgG second antibody to avoid conjugation of the second antibody with enzyme or radioisotopes. Monoclonal IgM was utilized as the capture antibody because IgM has greater avidity for specific antigen and a greater affinity towards plastics than IgG (140). Furthermore, the commercially available rabbit anti-mouse IgG conjugate does not cross-react with mouse IgM, but goat anti-mouse IgM will cross-react with mouse IgG.

Each step of the ds-ELISA which involved the use of immunochemicals was optimized. The optimal concentration of a particular immunochemical is that concentration which produces the highest positive response (all reactants present) and the lowest negative response (all reactants present except virus). This relationship is represented by P/N and is calculated by dividing the positive response by the negative response. The buffers and reagents utilized throughout the optimization procedures have been described previously under the indirect ELISA procedure. The additional buffer neccessary to perform the ds-ELISA is described below.

Binding buffer (0.05M carbonate, pH 9.6) This high pH buffer was used to non-covalently bind mouse monoclonal IgM to wells of a microtitration plate. Binding buffer was prepared as follows:

Na_2CO_3	0.16 g
$NaHCO_3$	0.29 g
ddH_2O up to	100 ml

If neccessary, adjust the pH to 9.6 with 1.0N NaOH or 1.0N HCl.

Optimization procedure Unless stated otherwise, each step of the ds-ELISA was incubated in a humidified chamber for 1 hr at room temperature or overnight at 4 C.

The plate wash procedure was performed as described for indirect ELISA.

The concentration of capture antibody was optimized by performing a serial dilution of purified IgM from 1:100 to 1:1600 in binding buffer. Each dilution was used to coat 8 wells of a 96-well microtitration plate at 50 ul/well. The plate was incubated and washed as described. Unbound protein sites were blocked by completely filling each well of the microtitration plate with BLOTTO. The plate was incubated and washed as described. Then, homologous virus, diluted 1.0 ug/ml in PBS, was added (50 ul/well) to 4 wells of each capture antibody dilution. To the other 4 wells, PBS (50 ul/well) was added as a negative control. The plate was incubated and washed 3 times. The immobilized virus was further reacted with 50 ul/well of purified IgG diluted 1:100 in wash buffer. The plate was incubated and washed 3 times. The amount of IgG bound to immobilized virus was detected by adding 50 ul/well of alkaline phosphatase conjugated rabbit anti-mouse IgG diluted 1:1000 in wash buffer with 1.0 % BSA. The plate was incubated and washed 3 times. At this time the colorimetric reaction was initiated by adding 50 ul/well of disolved substrate warmed to 37 C. The A_{410} of each well was measured in a plate Minireader II. The P/N ratio was calculated from the mean value of 4

identical wells.

The optimal concentration of IgG was determined by the same procedure, using the optimized concentration of IgM capture antibody. Likewise, the enzyme conjugated rabbit anti-mouse IgG was optimized using the calculated optimal concentrations of IgM and IgG, with enzyme conjugated antibody dilutions of 1:250 to 1:8000 in wash buffer with 1.0 % BSA.

Sample preparation for ds-ELISA

The detection of virus in infected sap was highly dependant upon the grinding buffer utilized during sample preparation. Therefore, several grinding buffers were tested to determine which would produce the most reliable results.

Approximately 0.5 g of infected or healthy corn tissue was macerated in a mortar and pestle with 4.5 ml (1:10 dilution) of grinding buffer. The buffers utilized to macerate the samples were binding buffer and wash buffer, each prepared with and without 2.0 % BSA. The plant sap was diluted 1:20 in the respective grinding buffer. Each diluted sample was added (50 ul/well) to 4 wells of a microtitration plate previously coated with capture antibody and blocked with BLOTTO. The plate was incubated overnight at 4 C and washed 3 times the follow-

ing day. The assay was completed as described previously using optimized concentrations of IgG and enzyme conjugated rabbit anti-mouse IgG. The A_{410} of each well was measured and values for the same treatment were averaged. P/N ratios were calculated and the grinding buffer which produced the highest mean value was used to prepare samples for each assay.

Assay of field isolates for the presence of MDMV

Virus-infected plants collected from areas of Iowa, Ohio, and Nebraska were generously donated to test the accuratcy of the McAb based ds-ELISA by John Hill and Helen Benner (ISU), Roy Gingery (Ohio Agricultural Research and Development Center), and Stanley Jensen (University of Nebraska), respectively. Microtitration plates previously coated with capture antibody and blocked with BLOTTO were shipped to each out of state cooperator.

Approximately 0.3-0.5 g of symptomatic tissue was macerated in a mortar and pestle with 2.7-4.5 ml of grinding buffer (sap:buffer dilution 1:10) determined as optimal for use with the MDMV-A specific ds-ELISA. The macerated leaf tissue was removed and 50 ul of plant sap was placed into each of 2 wells in the MDMV-A specific ds-ELISA and 2 wells in the MDMV-B specific ds-ELISA. The procedure was repeated with the same sample, grinding the

tissue in buffer determined optimal for the MDMV-B specific ds-ELISA. The ds-ELISA was performed as described and the strain of each MDMV infected plant and any cross-reactivity observed was recorded.

Strain identification of MDMV isolates infecting field samples obtained in Iowa was also assessed through the inoculation of indicator hosts. Each isolate was inoculated to 3 Johnsongrass seedlings as described previously. Viral replication within the symptomless Johnsongrass host was allowed to progress for 30 days. At this time, each Johnsongrass plant was back-inoculated to 2 Golden Bantam corn seedlings. Symptom development on the corn plants was observed and recorded over a 30 day period. Original isolates which ellicited symptoms on the back-inoculated Golden Bantam were concluded as being infected with MDMV-A. Those isolates which did not ellicit symptoms on back-inoculated Golden Bantam could be infected with one of several different strains of MDMV including MDMV-B.

Identification of isolates from Ohio, Nebraska, and Texas had been established by previous investigators.

RESULTS

Production of Monoclonal Antibodies

Two fusions were performed, using MDMV-A as one immunogen and MDMV-B as the other. A total of 1187 wells in 96-well tissue culture plates were cultured from the two fusions. Several hybridomas were observed after 3 days and by day 7 post-fusion 1001 or approximately 84 % of the wells contained hybridoma colonies. The hybridomas were assayed for specific antibody production by indirect ELISA using the original immunogen as the coating antigen. A total of 746 or approximately 75 % of the hybridomas secreted specific antibody. Fifty positive hybridomas from the MDMV-A fusion were cloned immediately.

The cross-reactivity of the MDMV-B positive hybridomas was assessed and those which cross-reacted with the heterologous MDMV-A strain were discarded. Only 18 of the original 260 (7 %) MDMV-B positive hybridomas did not cross-react with MDMV-A. These hybridomas were cloned immediately. The cloning procedure was very successful as 26 of the original 50 (52 %) MDMV-A positive hybridomas and 11 of the original 18 (61 %) MDMV-B specific hybridomas were successfully established and frozen. An additional 15 MDMV-A positive hybridomas were successfully

established, but subsequently contaminated during the freezing procedures; thus the cloning efficiency was approximately 71 %.

Established, McAb-producing, cell lines were given three letter designations followed by a roman numeral. The first two letters represent the original immunogen used to produce and screen the cell line; MA indicating that MDMV-A was the immunogen and MB indicating that MDMV-B was the immunogen. The third and final letter represents the antibody class secreted by the cell line; M for IgM and G for IgG. The three letters are followed by a Roman numeral to distinguish cell lines which have the same three letter designation. Therefore, a cell line which was cloned from the MDMV-B fusion and secretes IgG could be designated as MBG III. Table 1 gives a complete listing of the established McAb producing cell lines derived against MDMV.

Immunoblot Analysis of Monoclonal Antibodies

Immunoblot analysis of each McAb-producing cell line was utilized to demonstrate epitope specificity of the McAbs. I assumed that different immunoblot protein binding patterns are indicative of epitypically distinct McAbs.

Table 1. Cell line designations, monoclonal antibody subclass and light chain secreted, and cross-reactivity as determined by indirect ELISA

Cell line	IgG subclass	Light chain	Cross-reactivity[a]
MAGI	2a	K	++
MAGII	2a	K	-
MAGIII	NT[b]	K	NT
MAMI	--[c]	NT	+
MAMII	--	NT	NT
MAMIII	--	NT	NT
MAMIV	--	NT	-
MAMV	--	NT	-
MAMVI	--	NT	-
MAMVII	--	NT	-
MAMVIII	--	NT	++
MAMIX	--	NT	-
MAMX	--	NT	+/-
MAMXI	--	NT	+/-
MAMXII	--	NT	-
MAMXIII	--	NT	+
MAMXIV	--	NT	-
MAMXV	--	NT	+
MAMXVI	--	NT	NT
MAMXVII	--	NT	-
MAMXVIII	--	NT	-
MAMXIX	--	NT	-
MAMXX	--	NT	NT
MAMXXI	--	NT	NT
MAMXXII	--	NT	NT
MAMXXIII	--	NT	NT
MBGI	2b	K	-
MBGII	2a	K	-
MBGIII	1	K	-

[a]The level of cross-reactivity is indicated by: - = none, +/- = lower than homologous, + = same as homologous, and ++ = greater than homologous.

[b]NT = cell line not tested.

[c]-- = not applicable to this cell line.

Table 1 (Continued)

Cell line	IgG subclass	Light chain	Cross-reactivity
MBGIV	2a	K	-
MBGV	1	K	-
MBGVI	3	K	-
MBMI	--	NT	-
MBMII	--	NT	-
MBMIII	--	NT	-
MBMIV	--	NT	-
MBMV	--	NT	-

The enzyme concentration and digestion time utilized in the immunoblot procedure were selected to maximize the molecular weight range of protein fragments. Mini-gel analysis of the V8 protease digested viral protein, shown in Figure 1, indicated that an enzyme concentration of 0.5 mg/ml with a digestion time of 4 hr yielded a sufficient number of bands for immunoblot analysis.

The immunoblot procedure was performed with those cell lines which do not cross-react with the heterologous strain (Table 1). A total of 6 different protein binding patterns were observed. Two produced by the MDMV-A specific cell lines; 3 were produced by the MDMV-B specific cell lines; and the condition media (CM) negative control also resulted in the production of protein bands. The protein binding patterns are illustrated in Figure 2 and the cell lines represented by these binding patterns are listed in Table 2.

Further experiments were performed (results not presented) to determine the origin of the C1 bands. Using molecular weight standards, it was established that all three bands have molecular weights greater than the viral protein subunit. Therefore, the bands are not of viral origin. The C1 binding pattern could be reproduced; however, if an immunoblot was performed using V8 protease as the antigen. Because a great majority of the cell

Figure 1. Optimization of V8 Protease concentration and digestion time. MDMV viral protein was digested with 0.5, 1.0 and 2.0 mg/ml of enzyme for 2, 4, or 6, hrs at each dilution. Analysis of the digested polypeptides was performed on a Bio-Rad mini-gel as described in materials and methods

Figure 2. Immunoblot analysis of MDMV-A (A1 and A2), MDMV-B (B1-B3) specific cell lines, and conditioned media control (C1). Viral proteins were digested with V8 Protease and the polypeptides were electrophoretically transferred to a nitrocellulose membrane and probed with specific McAb's as described in materials and methods. Undigested coat protein is represented by (➤). The McAb secreting cell lines to which the immunoblots correspond to are listed in Table 2

Table 2. Immunoblot binding patterns exhibited by monoclonal antibody producing cell lines

Cell line	Immunoblot pattern[a]
MAGII	A1
MAMIV	A1
MAMV	A1
MAMVII	A1
MAMIX	C1
MAMXII	A1
MAMXIV	A1
MAMXVII	C1
MAMXVIII	A2
MAMXIX	A1
MBGI	B1
MBGII	B1
MBGIII	B1
MBGIV	B1
MBGV	B1
MBGVI	B3
MBMI	B1
MBMII	B3
MBMIII	B2
MBMIV	B3
MBMV	B1

[a] Immunoblot patterns are illustrated in Figure 1.

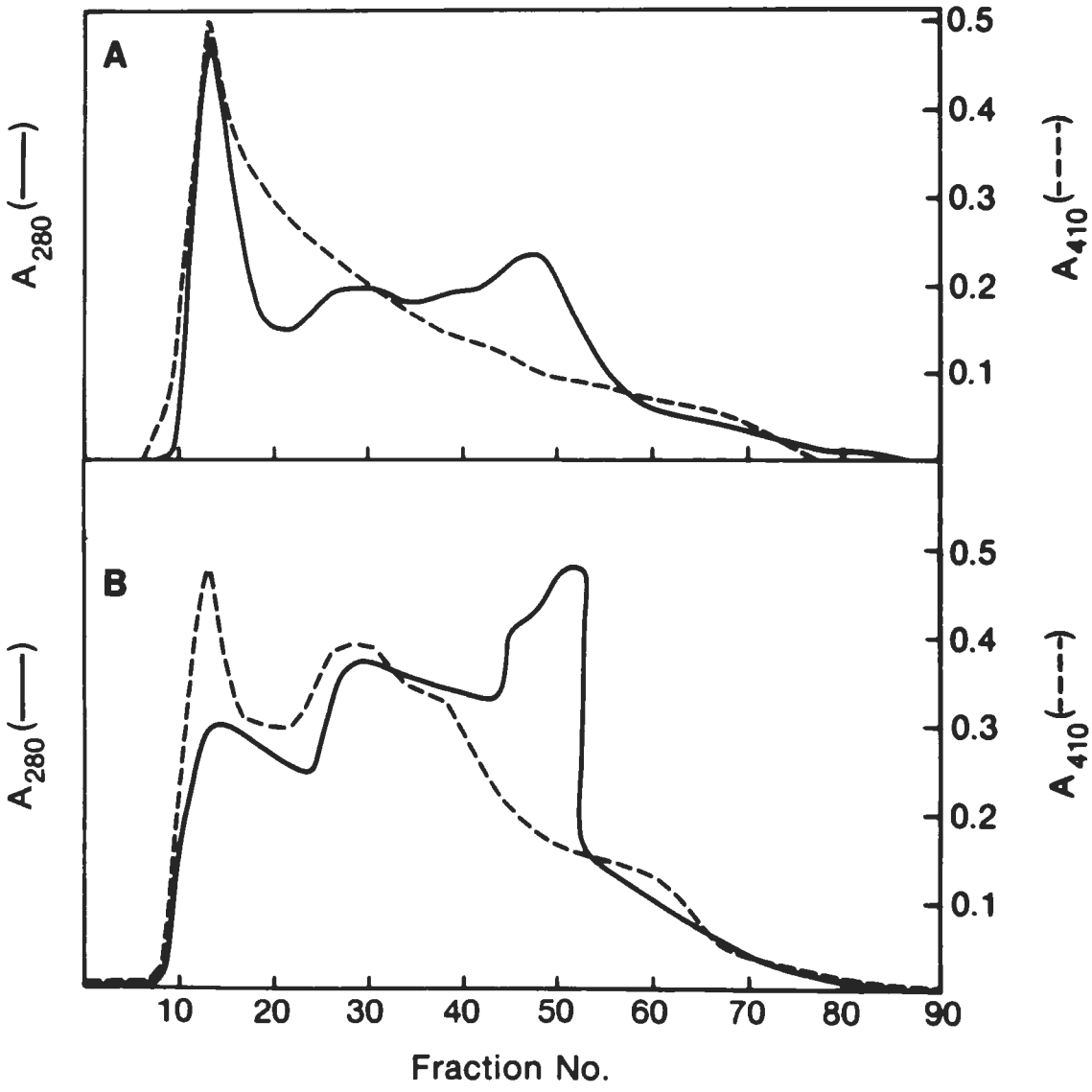
lines recognized the enzyme bands, it is more likely that the enzyme inhibits blocking of the nitrocellulose rather than specific recognition of the enzyme bands by the McAbs. These non-specific enzyme bands can be observed on the following blots: 1) upper band of A1, 2) upper three bands of A2, and 3) upper two bands of B1. These bands may not be in the same positions as each blot was performed in different experiments.

Purification of Mouse Monoclonal IgM from Ascitic Fluid

MDMV-A and MDMV-B specific mouse monoclonal IgM was purified from ascitic fluid produced by injection of Balb/c mice with the cell lines MAMXVIII and MBMIII, respectively. The IgM McAb was isolated from ascitic fluid by boric acid precipitation and further purified by Sepharose gel filtration.

Boric acid precipitation of 10 ml of ascitic fluid yielded approximately 6.5 mg of protein as determined by using $A_{280}^{0.1\%} = 1.4$. The entire boric acid-treated preparation was applied to a Sepharose CL-6B gel filtration column. The 35 ml void volume was discarded and 1.0 ml fractions were collected. The elution profile and the specific antibody content of each fraction (as determined by indirect ELISA) are depicted in Figure 3.

Figure 3. Elution profiles and antibody activity of Sepharose CL-6B purified monoclonal IgM. A) Sepharose elution profile of MDMV-A specific IgM (MAMXVIII; ——) and MDMV-A specific antibody activity of each eluted fraction as determined by indirect ELISA (– – –). B) Sepharose elution profile of MDMV-B specific IgM (MBMIII; ——) and MDMV-B specific antibody activity as determined by indirect ELISA (– – –)



Fractions 4 through 57 containing purified MAMXVIII and fractions 10 through 57 containing purified MBMIII were each pooled in 5.0 ml aliquots and the protein content of each aliquot was estimated using $A_{280}^{0.1\%} = 1.4$. All pooled samples were stored at -20 C until use. Table 3 and 4 list the protein content of each tube.

Purification of Mouse Monoclonal IgG from Ascitic Fluid

MDMV-A specific and MDMV-B specific IgG was purified from ascitic fluid produced by the injection of Balb/c mice with MAGII and MBGI, respectively. A 5.0 ml ascitic fluid sample, containing monoclonal IgG, was diluted 1:1 with binding buffer and applied to the an Affi-gel Protein A column (Bio-Rad). Fractions containing contaminating protein were discarded. When the absorbance at 280 nm was zero, elution buffer was added directly to the column and the 1.0 ml fractions of the IgG peak were collected into test tubes containing 2.0 ml of 1.0M Tris-HCl pH 9.0. The protein A elution profiles of MAGII and MBGI are presented in Figure 4. Fractions containing specific IgG (as determined by indirect ELISA) were pooled in 5.0 ml aliquots and the protein content of each aliquot was estimated using $A_{280}^{0.1\%} = 1.4$. The results obtained are shown in Tables 5 and 6.

Table 3. Protein content of Sepharose CL-6B eluted fractions containing MDMV-A specific IgM (MAMXVIII)

Tube	Eluted fractions[a]	A_{280}	Protein content (ug/ml)[b]
1	4-9	0.783	559
2	10-15	0.455	325
3	16-21	0.414	295
4	22-27	0.440	314
5	28-34	0.430	307
6	35-40	0.498	355
7	41-46	0.459	327
8	47-52	0.227	162
9	53-57	0.139	99

[a]Fractions containing MDMV-A specific IgM, as determined by indirect ELISA, were pooled and stored in 5.0 ml volumes.

[b]Protein content of each tube was estimated using $A_{280}^{0.1\%} = 1.4$.

Table 4. Protein content of Sepharose CL-6B eluted fractions containing MDMV-B specific IgM (MBMIII)

Tube	Eluted fractions[a]	A_{280}	Protein content (ug/ml)[b]
1	10-15	0.584	417
2	16-20	0.302	216
3	21-25	0.567	405
4	26-30	0.759	542
5	31-35	0.553	395
6	36-40	0.411	294
7	41-45	0.415	296
8	46-50	0.440	314
9	51-55	0.377	269
10	56-57	0.288	206

[a]Fractions containing MDMV-B specific IgM, as determined by indirect ELISA, were pooled and stored in 5.0 ml volumes.

[b]Protein content of each tube was estimated using $A_{280}^{0.1\%} = 1.4$.

Figure 4. Elution profiles of MDMV-A specific (MAGII; A) and MDMV-B specific (MBGI; B) protein A purified monoclonal IgG. Arrow indicates point of IgG elution with elution buffer

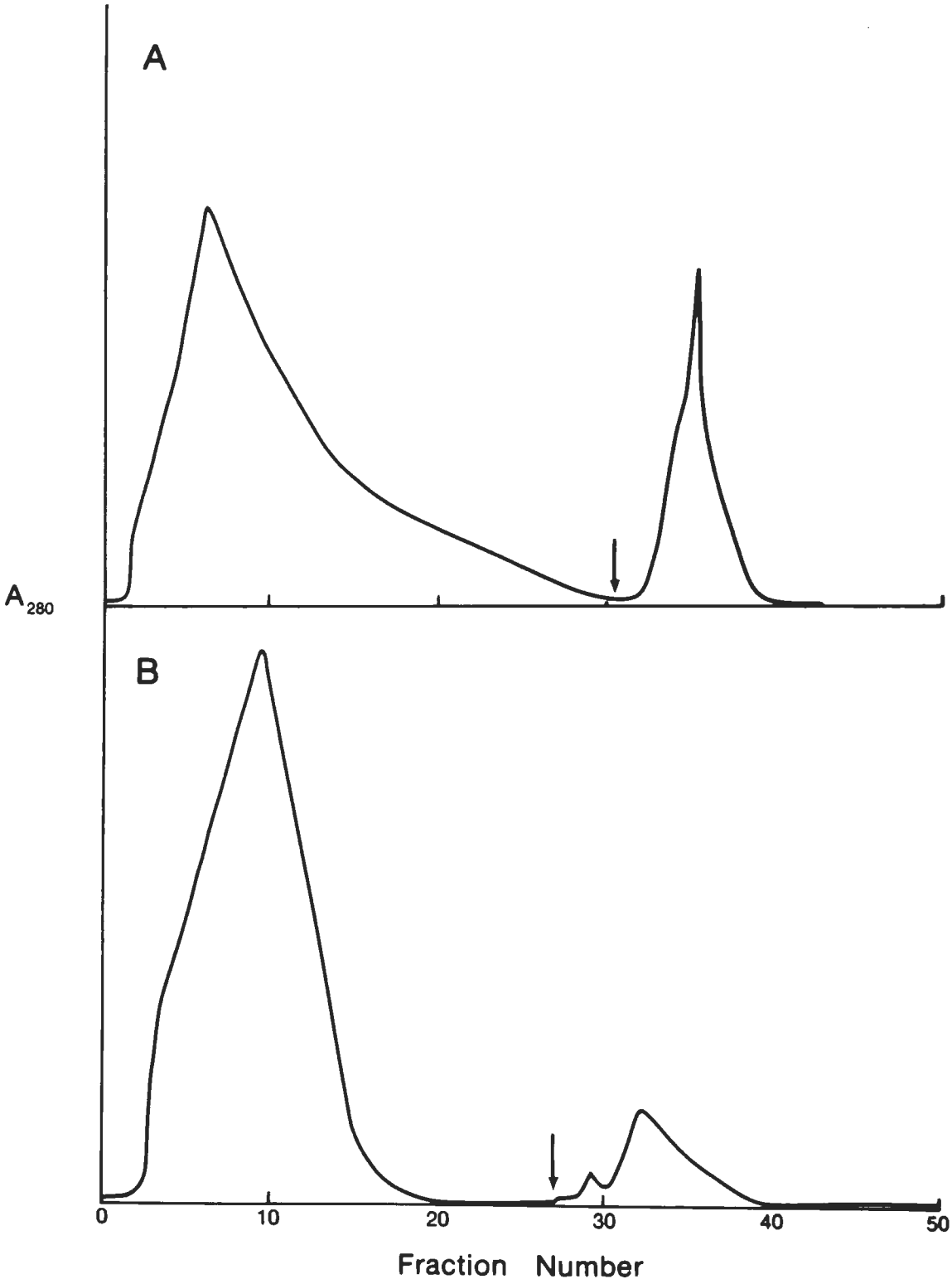


Table 5. Protein content of Protein A purified fractions containing MDMV-A specific IgG (MAGII)

Tube	A_{280}	Protein content (mg/ml)[a]
1	0.495	0.354
2	2.082	1.490
3	1.800	1.290
4	1.789	1.280
5	1.113	0.795
6	0.213	0.152
7	0.086	0.061
8	0.038	0.027
9	0.023	0.016
10	0.028	0.020
11	0.014	0.010

[a]Protein content of each tube was estimated using $A_{280}^{0.1\%} = 1.4$.

Table 6. Protein content of Protein A purified fractions containing MDMV-B specific IgG (MBGI)

Tube	A_{280}	Protein content (ug/ml)[a]
1	0.259	185
2	0.600	429
3	0.606	433
4	0.424	303
5	0.231	165
6	0.094	67
7	0.048	34
8	0.021	15
9	0.017	12
10	0.005	4
11	0.004	3
12	0.004	3
13	0.004	3

[a]Protein content of each tube was estimated using $A^{0.1\%}_{280} = 1.4$.

Optimization of Monoclonal Antibody Based ds-ELISA

The concentrations of capture antibody, second antibody, and enzyme conjugated-antibody were optimized for the specific detection of MDMV-A and MDMV-B. Dilutions of each reagent were prepared and utilized in the ds-ELISA as described. The A_{410} of each well was measured and the P/N ratios calculated for capture antibody, second antibody, and enzyme conjugated antibody are expressed graphically in Figures 5, 6, and 7, respectively.

Results indicate that the optimal dilutions of capture antibody (MAMXVIII), second antibody (MAGII), and enzyme conjugated antibody for the specific detection of MDMV-A are 1:800, 1:200, and 1:2000, respectively. These dilutions correspond to protein concentrations of 0.699, 7.45, and 0.450 ug/ml, respectively. The optimal dilutions of capture antibody (MBMIII), second antibody (MBGI), and enzyme conjugated antibody for the specific detection of MDMV-B are 1:1600 (highest dilution tested), 1:400, and 1:2000, respectively. These dilutions correspond to protein concentrations of 0.260, 1.07, and 0.450 ug/ml, respectively.

Figure 5. Determination of the optimal capture antibody dilution for the MDMV-A specific ds-ELISA (□—□) and the MDMV-B specific ds-ELISA (▲—▲). Purified monoclonal IgM (MAMXVIII and MBMIII, respectively) was diluted from 1:100 to 1:1600 and utilized in the ds-ELISA as described in materials and methods. The A_{410} in the presence (P) and absence (N) of virus was measured. P/N ratios were calculated and plotted. Data points are the mean values of duplicate experiments

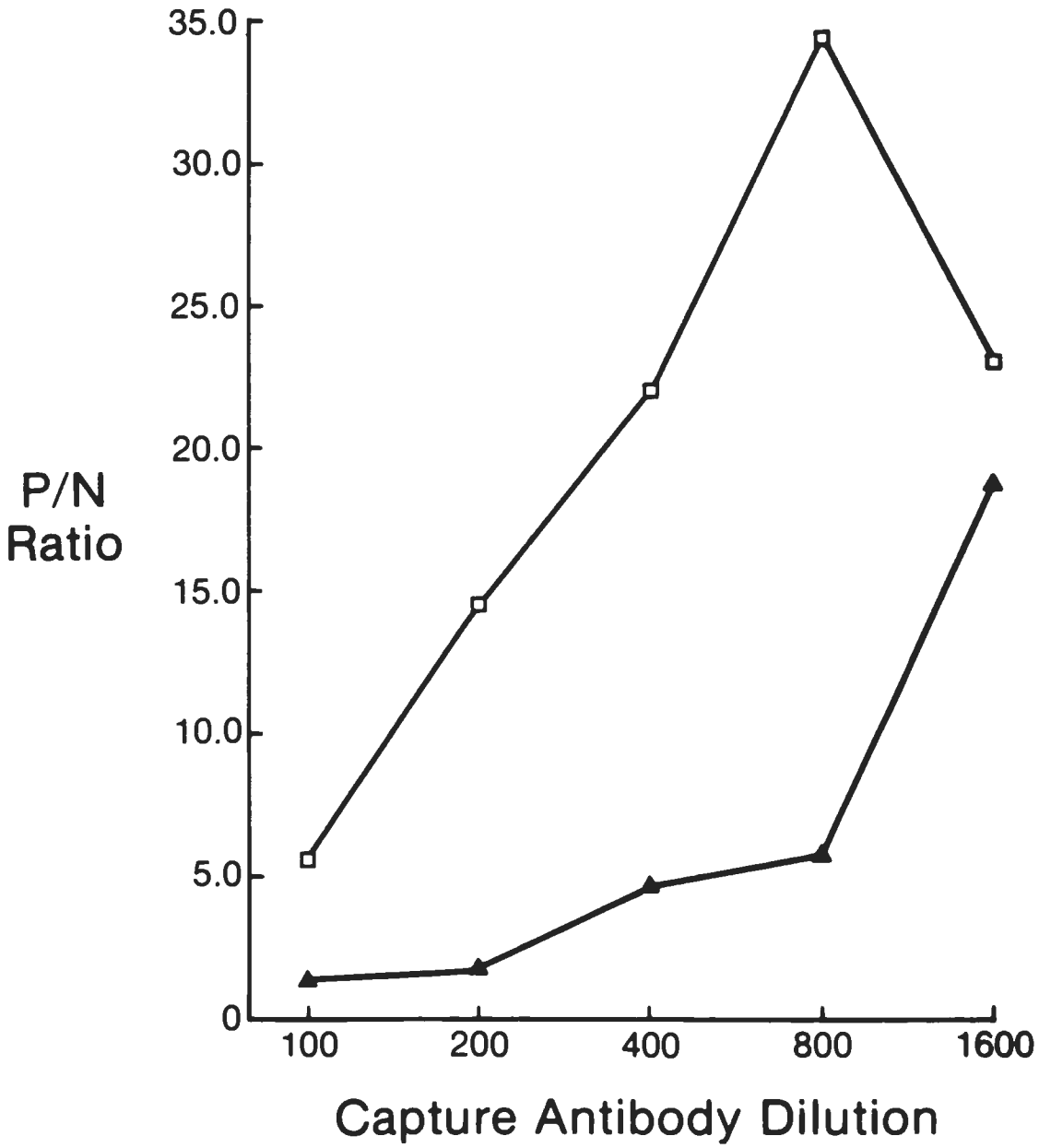


Figure 6. Determination of the optimal second antibody dilution for the MDMV-A specific ds-ELISA (□—□) and the MDMV-B specific ds-ELISA (▲—▲). Purified monoclonal IgG (MAGII and MBGI, respectively) was diluted from 1:100 to 1:1600 and utilized in the ds-ELISA as described in materials and methods. The A_{410} in the presence (P) and absence (N) of virus was measured. P/N ratios were calculated and plotted. Data points are the mean values of duplicate experiments

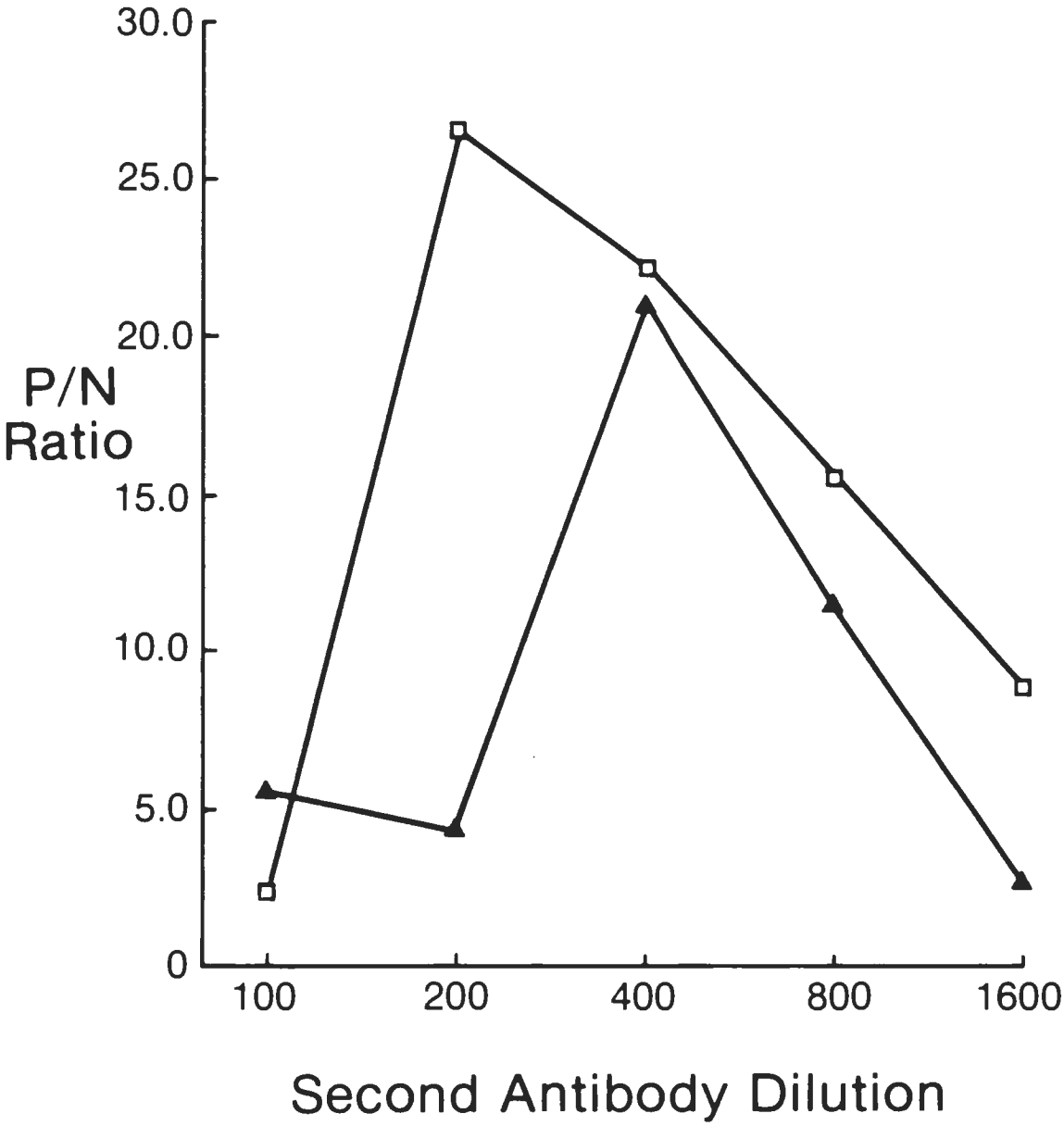
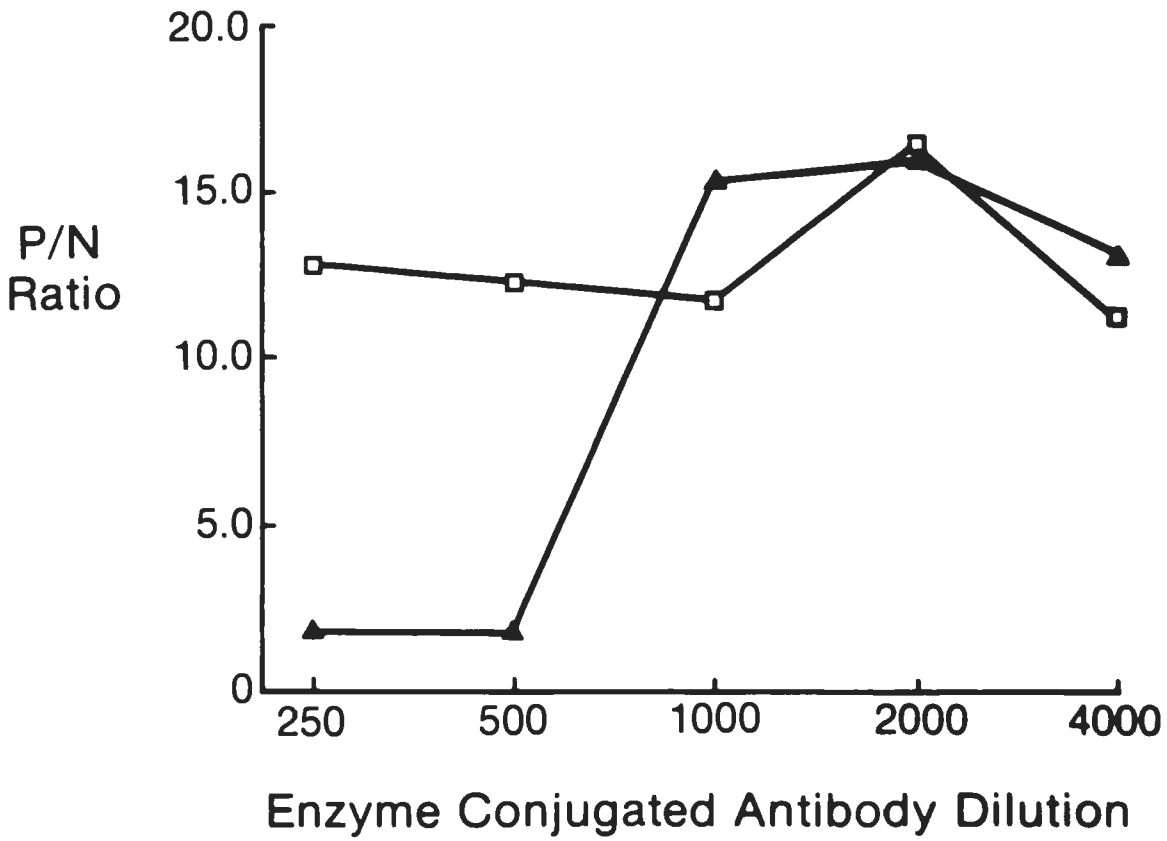


Figure 7. Determination of the optimal enzyme conjugated antibody dilution for the MDMV-A specific ds-ELISA (□—□) and the MDMV-B specific ds-ELISA (▲—▲). Commercially available alkaline phosphatase conjugated rabbit anti-mouse IgG antibody (Sigma A-1902) was diluted from 1:250 to 1:4000 and utilized in the ds-ELISA as described in materials and methods. The A_{410} in the presence (P) and absence (N) of virus was measured. P/N ratios were calculated and plotted. Data points are the mean values of duplicate experiments



Sensitivity of Optimized ds-ELISA Using Purified Virus

The sensitivity of each ds-ELISA was determined with dilutions of purified homologous virus from 1000 ng/ml (50 ng/well) to 1.0 ng/ml (50 pg/well). In addition, non-specific detection of the heterologous virus was assessed for each ds-ELISA using dilutions of purified heterologous virus from 10,000 ng/ml (500 ng/well) to 10 ng/ml (500 pg/well). A reaction was considered positive if the mean A_{410} was greater than 0.10.

Using optimized conditions, the detection sensitivity of the MDMV-A specific ds-ELISA was between 400 and 800 pg/well or 8 and 16 ng/ml (Figure 8). In addition, no cross-reactivity was observed with the heterologous strain MDMV-B. The detection sensitivity of the optimized MDMV-B specific ds-ELISA was 1.6 ng/well or 32 ng/ml (Figure 9) and no cross-reactivity was observed with the heterologous strain MDMV-A.

Sample Preparation for ds-ELISA

The specific detection of virus in infected plant samples was highly dependent upon the grinding buffer

Figure 8. Detection sensitivity of MDMV-A specific ds-ELISA. Purified MDMV-A (□—□) was diluted from 1000 to 1.0 ng/ml in PBS and the heterologous virus, MDMV-B (▲—▲) was diluted from 10,000 to 1.0 ng/ml in PBS. Each dilution was applied to a microtitration plate previously coated with MDMV-A specific capture antibody (MAMXVIII) and blocked with BLOTTO. The assay was completed as described in materials and methods and the mean A_{410} value of identical samples was calculated and plotted. A mean value greater than 0.10 was interpreted as a positive. Data points are the mean values of duplicate experiments

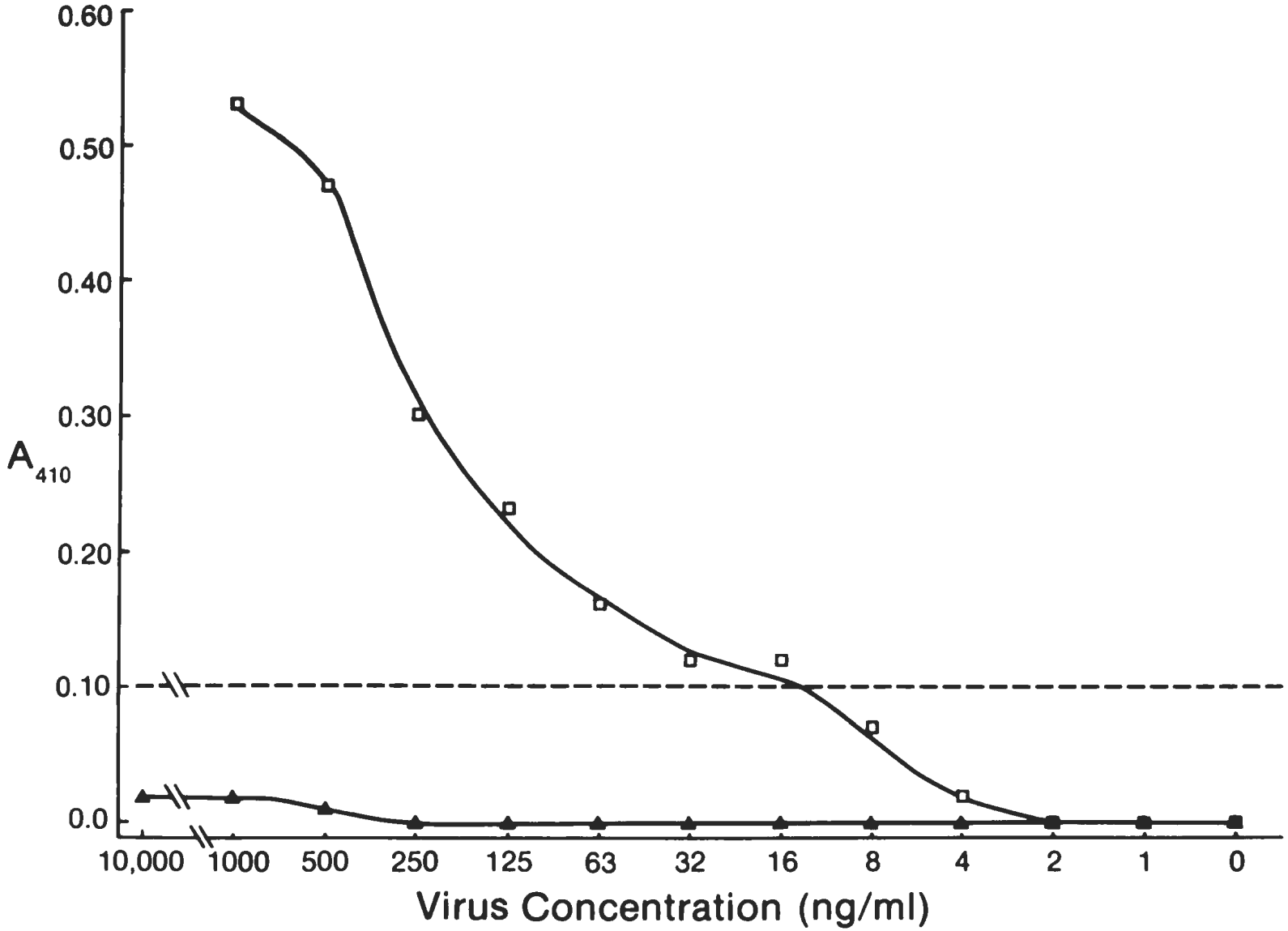
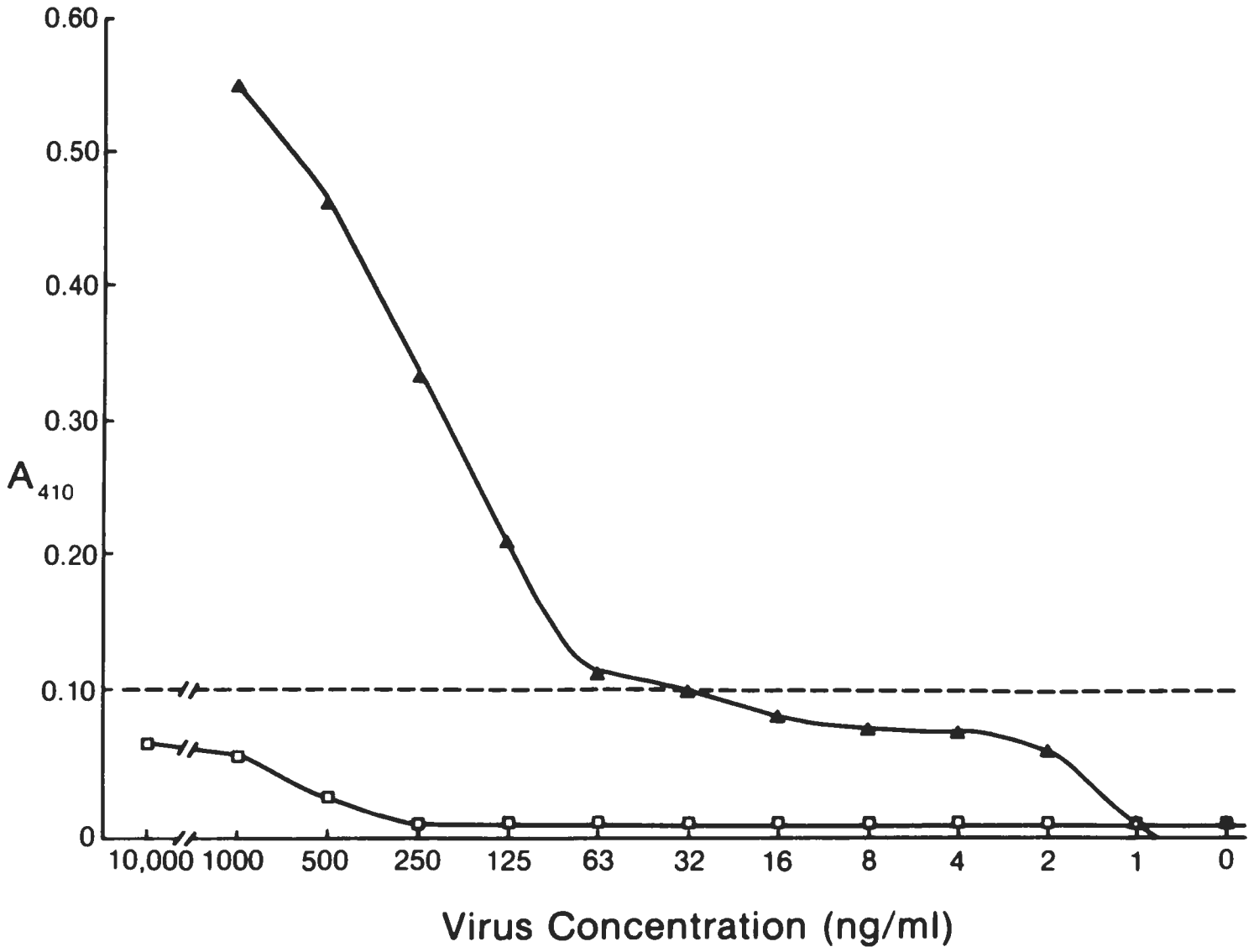


Figure 9. Detection sensitivity of MDMV-B specific ds-ELISA. Purified MDMV-B (□—□) was diluted from 1000 to 10 ng/ml in PBS and the heterologous virus, MDMV-A (▲—▲), was diluted from 10,000 to 1.0 ng/ml in PBS. Each dilution was applied to a microtitration plate previously coated with MDMV-B specific capture antibody (MBMIII) and blocked with BLOTTO. The assay was completed as described in materials and methods and the mean A_{410} value of identical samples was calculated and plotted. A mean value greater than 0.10 was interpreted as a positive. Data points are the mean values of duplicate experiments



utilized during sample preparation. Sample preparation for each ds-ELISA was tested using 0.05M carbonate buffer, pH 9.6 (binding buffer), and 0.01M PBS, pH 7.2, containing 0.05 % Tween 20; each prepared with and without 2.0 % BSA. Negative controls included corn tissue infected with the heterologous strain and healthy corn tissue.

The results, presented in Table 7, indicate that 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA is the optimal grinding buffer when preparing samples for use in the MDMV-A specific ds-ELISA. A positive response (A_{410} 0.1 greater than PBS control) was also obtained with 0.05M carbonate buffer, pH 9.6; however, the addition of 2.0 % BSA to this buffer increased the positive response almost 3-fold. The results, presented in Table 8, indicate that 0.01M PBS, pH 7.2, containing Tween 20 and 2.0 % BSA is the optimal grinding buffer when preparing samples for use in the MDMV-B specific ds-ELISA. The presence of BSA in this buffer gave a positive response 1.5 times greater than 0.01M PBS, pH 7.2, containing Tween 20.

Table 7. Assay of MDMV infected and healthy corn tissue prepared in various grinding buffers for use in the optimized MDMV-A specific ds-ELISA

	Grinding buffers[a]			
Tissue sample	PBS-T	PBS-T-BSA	Carbonate	Carbonate-BSA
Healthy	0.005[b]	0.005	0.003	0.000
MDMV-B infected	0.008	0.000	0.000	0.000
MDMV-A infected	0.015	0.008	0.140	0.405
PBS control	0.000	0.000	0.000	0.000

[a]Grinding buffers included: PBS-T = 0.01M PBS, pH 7.2, containing 0.05 % Tween 20; PBS-T-BSA = 0.01M PBS, pH 7.2, containing 0.05 % Tween 20 and 2.0 % BSA; carbonate = 0.05M carbonate buffer, pH 9.6; carbonate-BSA = 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA.

[b]Results represented as the mean A_{410} value of identical samples. Values greater than 0.100 indicate positive samples.

Table 8. Assay of MDMV infected and healthy corn tissue prepared in various grinding buffers for use in the optimized MDMV-B specific ds-ELISA

	Grinding buffers[a]			
Tissue sample	PBS-T	PBS-T-BSA	Carbonate	Carbonate-BSA
Healthy	0.005[b]	0.000	0.000	0.000
MDMV-B infected	0.165	0.238	0.005	0.005
MDMV-A infected	0.000	0.003	0.000	0.005
PBS control	0.000	0.000	0.000	0.000

[a]Grinding buffers included: PBS-T = 0.01M PBS, pH 7.2, containing 0.05 % Tween 20; PBS-T-BSA = 0.01M PBS, pH 7.2, containing 0.05 % Tween 20 and 2.0 % BSA; carbonate = 0.05M carbonate buffer, pH 9.6; carbonate-BSA = 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA.

[b]Results represented as the mean A_{410} value of identical samples. Values greater than 0.100 indicate positive samples.

Sensitivity of Optimized ds-ELISA Using Infected Plant Tissue

The level of detection of each ds-ELISA was assessed using MDMV infected corn tissue. Tissue infected with the homologous strain of MDMV was macerated in a mortar and pestle and diluted from 1:10 to 1:5120 in grinding buffer. Negative controls included healthy corn tissue and tissue infected with the heterologous strain of MDMV, also diluted 1:10 to 1:5120 in grinding buffer. Grinding buffers utilized to prepare samples for the MDMV-A ds-ELISA and the MDMV-B ds-ELISA were 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA and 0.01M PBS, pH 7.2, containing 0.05 % Tween 20 and 2.0 % BSA, respectively.

The results, presented in Figure 10, indicate that the MDMV-B ds-ELISA can specifically detect virus in sap at dilutions between 1:2560 and 1:5120, whereas the MDMV-A ds-ELISA can only specifically detect virus in sap dilutions up to 1:40 (Figure 11). The low detection level exhibited by the MDMV-A ds-ELISA was thought to be related to pH as samples were diluted in the high pH carbonate buffer. This results in an increased pH as the dilution of sap increases. The effect of pH on the specific detection of MDMV-A was therefore determined.

Figure 10. Detection sensitivity of MDMV-B specific ds-ELISA using infected plant sap. MDMV-B infected (▲—▲), MDMV-A infected (□—□), and healthy (○—○) corn tissue were macerated in separate mortar and pestles with 0.01M PBS, pH 7.2, containing 0.05 % Tween 20 and 2.0 % BSA. Each preparation was diluted 1:10 to 1:5120 in the above buffer and applied to a microtitration plate previously coated with MDMV-B specific capture antibody (MBMIII) and blocked with BLOTTO. The assay was completed as described in materials and methods and the mean A_{410} value of identical samples was calculated and plotted. A mean value greater than 0.10 was interpreted as a positive. Data points are the mean values of duplicate experiments

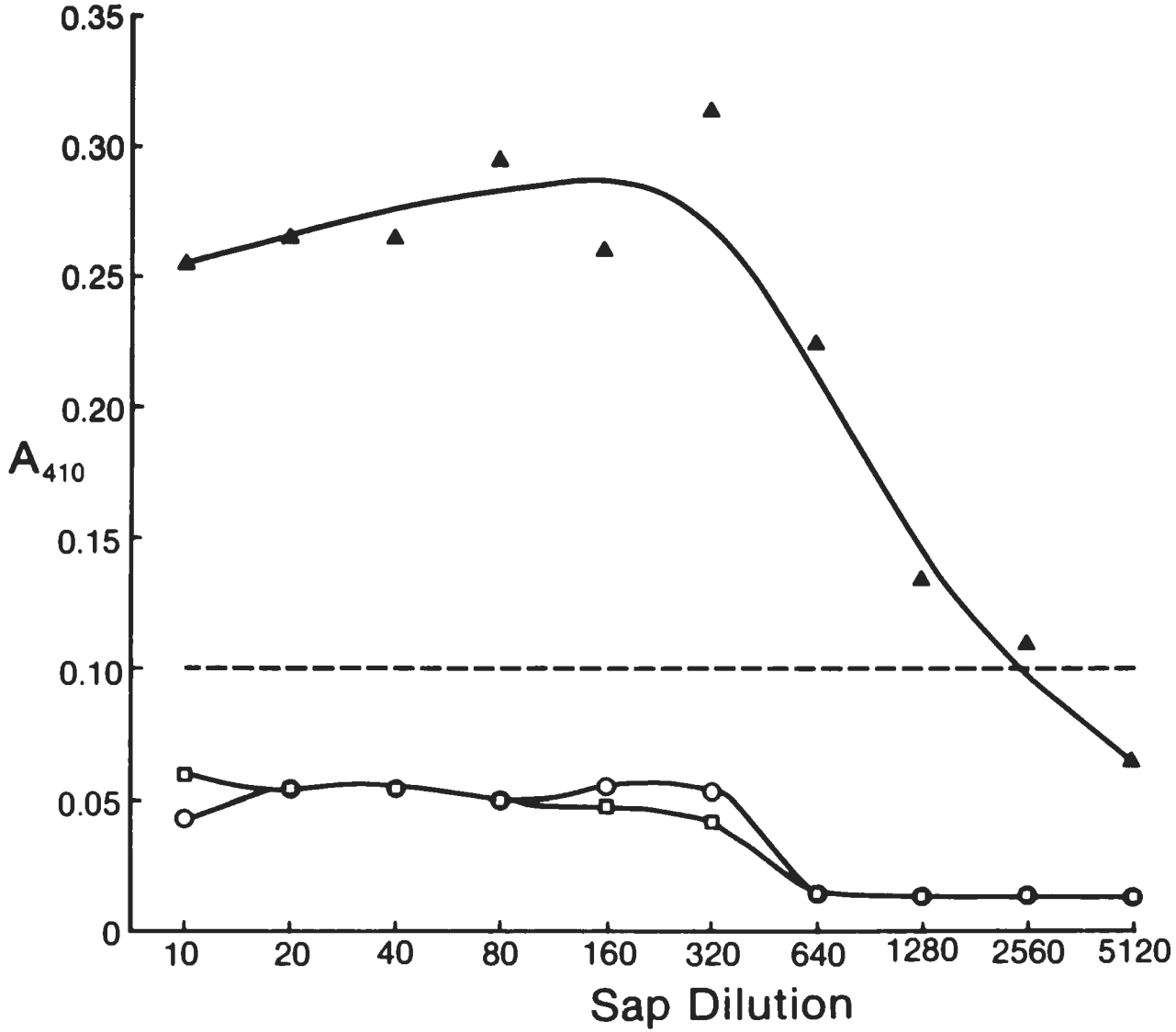
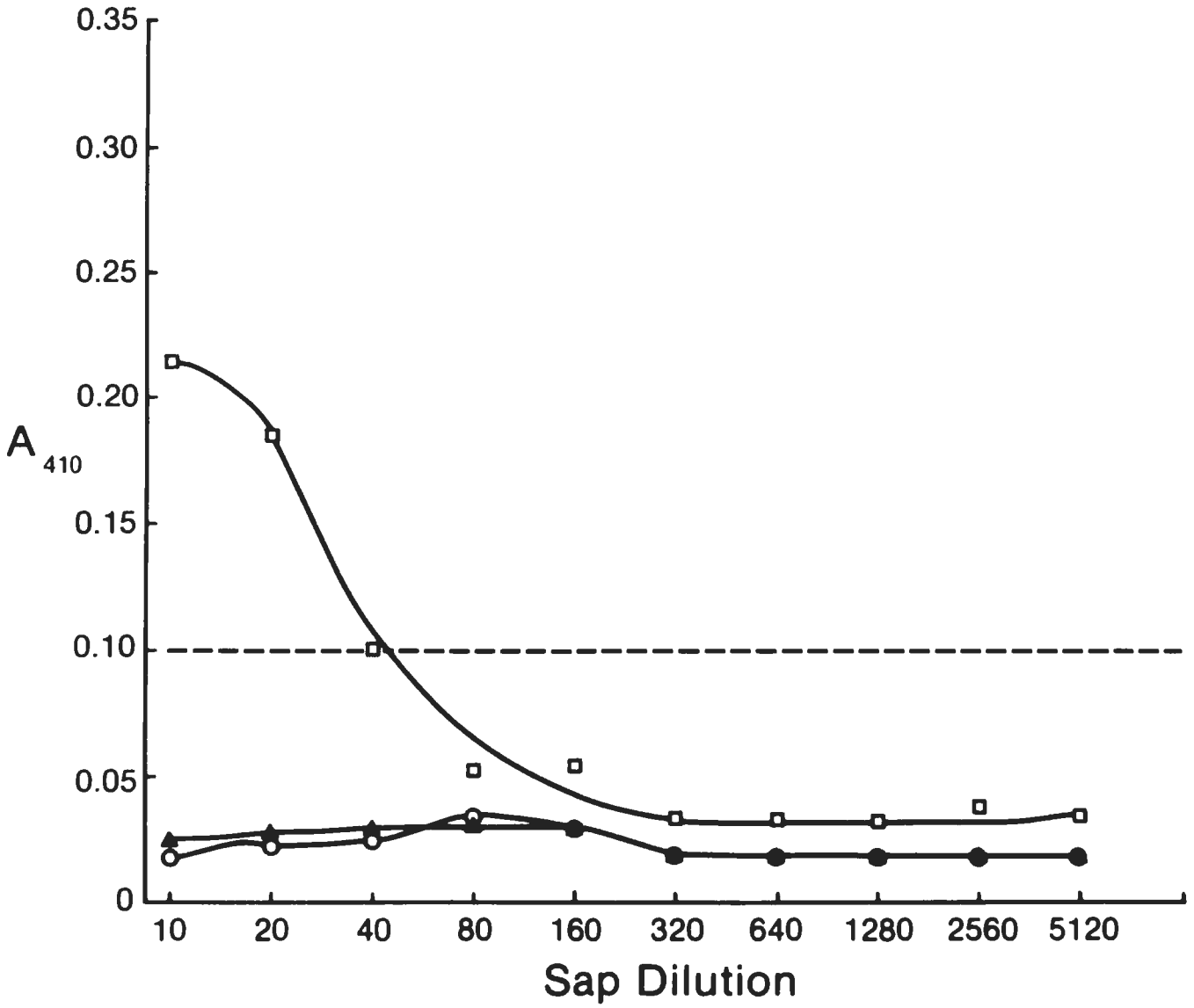


Figure 11. Detection sensitivity of MDMV-A specific ds-ELISA using infected plant sap. MDMV-A infected (□—□), MDMV-B infected (▲—▲), and healthy (○—○) corn tissue were macerated in separate mortar and pestles with 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA. Each preparation was diluted 1:10 to 1:5120 in the above buffer and applied to a microtitration plate previously coated with MDMV-A specific capture antibody (MAMXVIII) and blocked with BLOTTO. The assay was completed as described in materials and methods and the mean A_{410} value of identical samples was calculated and plotted. A mean value greater than 0.10 was interpreted as a positive. Data points are the mean values of duplicate experiments



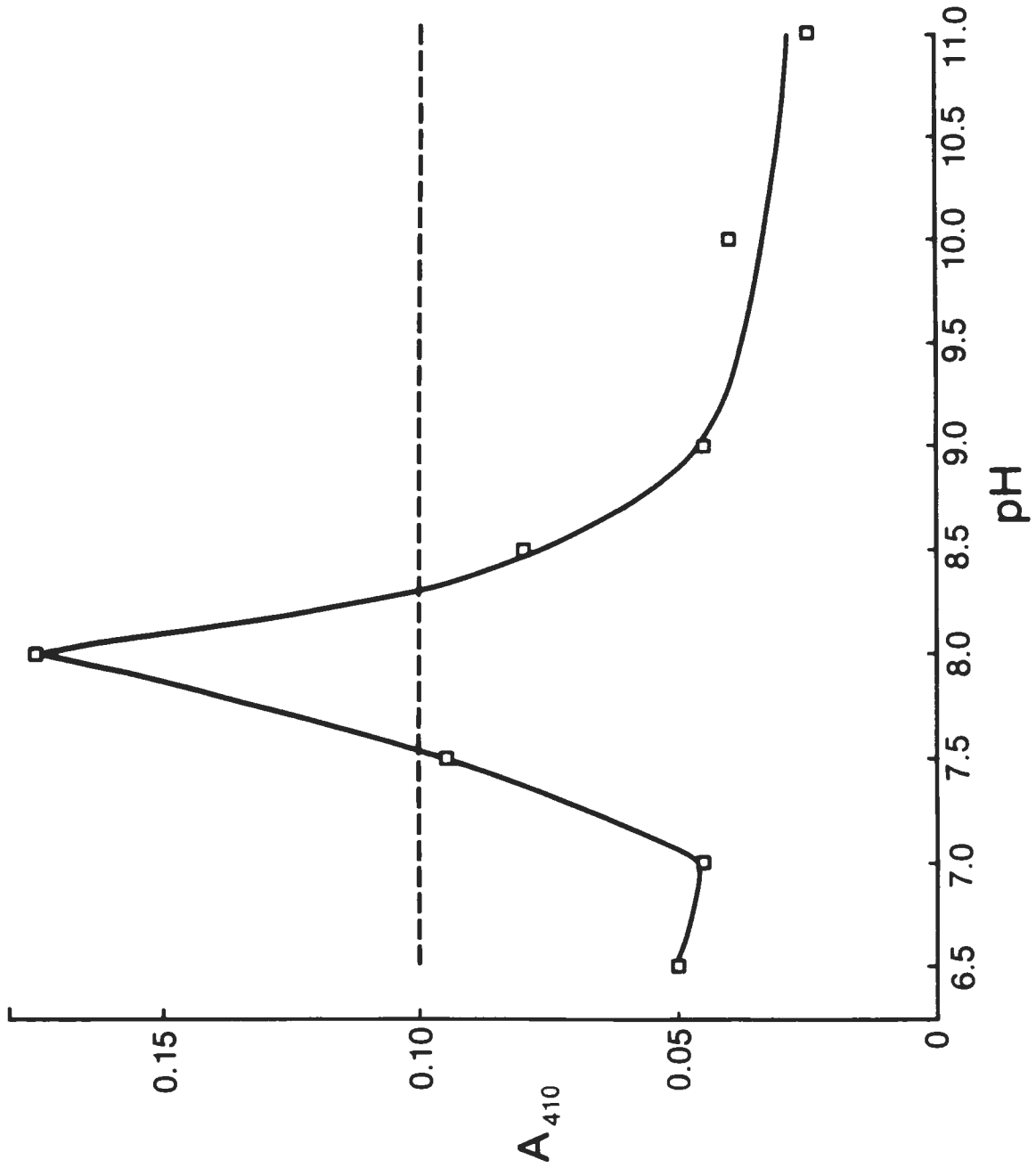
MDMV-A infected corn tissue was macerated in a mortar and pestle with PBS at a tissue:buffer ratio of 1:20. The plant sap was distributed into 8 tubes at 1.0 ml/tube. The first tube was set aside. One drop of 1.0N NaOH were added to the second tube and 2 drops of 1.0N NaOH was added to the third tube. This pattern was continued such that the eighth tube contained 7 drops of 1.0N NaOH. The pH of each tube was measured using pH paper (Micro Essential Laboratory, Brooklyn, NY) and two 50 ul samples from each tube were applied to 2 wells of a microtitration plate previously coated with MDMV-A specific capture antibody (MAMXVIII) and blocked with BLOTTO. The assay was completed, using procedures previously described, and the mean A_{410} values vs the sample pH was plotted.

The results presented in Figure 12 indicate that the MDMV-A specific ds-ELISA is pH dependent and that the optimal sample pH is between 7.5 and 8.5.

Assay of Field Isolates for the Presence of MDMV

Several microtitration plates coated with specific IgM capture antibody and blocked with BLOTTO were shipped via express mail to out-of-state cooperators. The cooperators applied virus infected corn samples to the plates using the appropriate grinding buffer and proce-

Figure 12. Effect of sample pH on the detection of MDMV-A in infected corn tissue. MDMV-A infected corn tissue was macerated in a mortar and pestle with PBS. One ml sap aliquots were adjusted to various pH values between 6.5 and 11.0 by addition of 1.0N NaOH. Each sample was applied to a microtitration plate previously coated with MDMV-A specific capture antibody (MAMXVIII) and blocked with BLOTTO. The assay was completed as described in materials and methods and the mean A_{410} value of identical samples was calculated and plotted. A mean value greater than 0.10 was interpreted as a positive. Data points are the mean values of duplicate experiments



dures described previously. The plates were returned and analyzed in our laboratory. Results obtained from the Iowa isolates and the 2 out-of-state cooperators are presented in Table 9.

Table 9. Assay of field isolates with MDMV-A specific and MDMV-B specific ds-ELISA's

Isolate strain and designation[b]	Isolate source[c]	ds-ELISA A_{410} values[a]	
		MDMV-A	MDMV-B
MDMV-B Asgrow 2	Hill	--	0.135
MDMV-B Asgrow 3	Hill	--	0.190
MDMV-B Asgrow 4	Hill	--	0.110
MDMV-B NK199 1	Hill	--	0.185
MDMV-B NK199 2	Hill	--	0.140
MDMV-B NK199 3	Hill	--	0.230
MDMV-B NK199 6	Hill	--	0.130
MDMV-B NK199 7	Hill	--	0.200
MDMV-B Silver Q 1	Hill	--	0.185
MDMV-B Silver Q 2	Hill	--	0.130
MDMV-B Silver Q 3	Hill	--	0.155
MDMV-B 41F	Hill	--	0.160
MDMV-B 51F	Hill	--	0.140
MDMV-B Ia 188	Hill	--	0.120
MDMV-A	Hill	0.260	--
MDMV-A	Jensen	0.340	--
MDMV-A Minn 6	Jensen	0.675	--
MDMV-A Minn 11	Jensen	0.540	--

[a] Each strain specific ds-ELISA (MDMV-A and MDMV-B) was performed as described in materials and methods using the appropriate grinding buffer. Results are represented as the mean A_{410} value of identical samples. Positive samples are those which have an A_{410} greater than 0.1. Negative samples are represented by --.

[b] Maize dwarf mosaic virus (MDMV) and sugarcane mosaic virus (SCMV) strains and designations were established by previous investigators.

[c] Infected isolates from Ankeny, Iowa, were obtained by John Hill and Helen Benner and maintained at Iowa State University (Hill). Microtitration plates previously coated with capture antibody and blocked with BLOTTO were shipped to the following out of state cooperatives for sample application: Stanley Jensen (University of Nebraska), and Roy Gingery (Ohio Agricultural Research and Development Center).

Table 9 (Continued)

Isolate strain and designation	Isolate source	ds-ELISA A_{410} values	
		MDMV-A	MDMV-B
MDMV-A Ohio D[d]	Jensen	0.390	--
MDMV-A Ohio E	Jensen	--	--
MDMV-A Ohio F	Jensen	--	--
MDMV-A Yazoo Miss	Jensen	0.120	--
MDMV-B	Jensen	--	1.205
MDMV-B Field B	Jensen	--	0.740
MDMV-B Ia 188	Jensen	--	0.120
MDMV-B Minn B	Jensen	--	0.460
MDMV-B Unknown	Jensen	--	--
MDMV-KS1	Jensen	--	--
SCMV-A	Jensen	--	0.275
SCMV-B	Jensen	--	--
SCMV-B Miss	Jensen	--	--
SCMV-H	Jensen	--	--
SCMV-M	Jensen	--	--
MDMV-A	Gingery	0.160	--
MDMV-B	Gingery	--	0.410
MDMV-D	Gingery	--	--
MDMV-E	Gingery	--	--
MDMV-F	Gingery	0.200	--
MDMV-O	Gingery	--	--

[d]Jensen refers to MDMV-A Ohio D, MDMV-A Ohio E, and MDMV-A Ohio F as substrains of MDMV-A; whereas, Gingery refers to these same isolates as MDMV strains D, E, and F, respectively.

DISCUSSION

Early attempts to produce McAb secreting hybridomas were not successful. Therefore, it was necessary to modify the fusion and cloning procedures of Van Deusen and Whetstone (149) and the immunization procedure described by Hill et al. (50). Significant modifications, which together resulted in highly successful fusions, included: 1) increasing the immunization time period and using less antigen, 2) decreasing the time of cell exposure to the fusogen PEG, 3) increasing the pH of the fusion media used to dilute cells following fusion, and 4) using a cloning procedure which foregoes cell culture scale-up prior to cloning.

The success of a particular fusion procedure was directly related to the specific serum titer of the immunized mouse. Use of the immunization procedure described by Hill et al. (50) resulted in a serum titer between 1:256 and 1:512 and the subsequent spleen cell fusion yielded very few hybridomas (2-10 % of the cultured wells). However, use of the immunization procedure described in this study, which requires half the antigen (100 ug vs. 200 ug) administered over a longer period of time (7-10 wks vs. 4-6 wks), resulted in serum titers of

1:12,000 or greater and the spleen yielded successful hybridomas in 50-70 % of the cultured wells. Furthermore, less than 1.0 % of the successful hybridomas obtained from the low titer mouse spleen, secreted immunogen specific antibody whereas 75 % of the successful hybridomas obtained from the high titer mouse spleen secreted immunogen specific antibody. One reason for the success of this immunization procedure may be the stimulation of dividing spleen cells, as these are the cells which give rise to viable hybridomas (157).

Although the extended immunization procedure greatly enhanced the cell fusion efficiency; certain modifications of the fusion procedure described by Van Deusen and Whetstone (149) were made to promote the production and successful cloning of a greater percentage of viable hybridomas. Significant modifications of the procedure included short-duration exposure of the fusion mixture to PEG and ultilization of fusion media at pH 8.0 rather than pH 7.2. These two modifications together increased the number of successful hybridomas from 50-70 % to 80-100 % of the cultured wells.

It has been shown that PEG exposure periods greater than 60 sec have a detrimental effect upon the number of viable hybridomas presumably due to the increased sensitivity of newly formed hybridomas to the cytotoxic

effects of PEG (81). Most reported fusion procedures recommend a total PEG exposure of 2.0 min or greater(36, 115, 149, 157, 158), which is well beyond the PEG tolerance level of newly formed hybridomas.

Another fusion procedure modification was an increase of the fusion media pH from 7.4 to 8.0. The effect of pH on mammalian cell fusion has been investigated by Johnson and Rao (71) and Rao and Johnson (114). They found that for the successful fusion of two cells, resulting in the formation of a binucleate cell, one of two phenomena must occur: 1) premature chromosome condensation (PCC) of the interphase nucleus or, 2) the formation of a nuclear envelope around the metaphase chromosomes. These occurrences are highly dependent upon environmental pH; a pH of 8.0 is optimal for both (71). Therefore, fusion media adjusted to this pH results in the maximal number of viable hybridomas during cell fusion procedures (157).

Using the optimal fusion conditions described above, a successful fusion resulted in an average of 500 cultured wells containing at least one hybridoma colony. Approximately 375 or 75 % of these colonies were found to secrete specific antibody. Westerwoudt et al. (158) have shown that 14 days post-fusion, only 35 % of the original positives tested 9 days post-fusion, still produced specific antibody. Therefore, it was apparent that a

rapid cloning method should be employed such that antibody secreting hybridomas would be maintained.

Several methods exist for the cloning of hybridoma cells including limiting-dilution (36, 103), use of solid-phase supports (4, 149), and single-cell transfer (37). However all of these procedures are time-consuming and result in the loss of a majority of the antibody producing cell lines. The rapid cloning procedure developed in our laboratory foregoes the time consuming scale-up procedure prior to cloning. Therefore, hybridomas were cloned directly from 96-well tissue-culture plates with a successful cloning efficiency of 71 %.

Cell line instability is another major problem commonly encountered during hybridoma production. This is a result of the temporary increase in chromosome number after fusion and the eventual random elimination of chromosomes in the hybrid cells. The elimination of chromosomes may result in cell death if genes essential for growth in tissue culture are lost, or, more commonly, loss of antibody production.

In this study, loss of antibody production was observed during cell cloning procedures and with established cell lines maintained for extended periods in continuous culture. The cloning procedure resulted in a 29 % loss of viable hybridomas. None of the hybridomas failed to grow;

rather, the subclones did not secrete or produce antibody. Furthermore, several cloned and established cell lines ceased to produce antibody after continuous culture of 2 weeks or longer. Loss of antibody production at this stage is probably not due to the loss of antibody producing chromosomes, but may be a result of improper regulation (113), failure of a cell line to secrete immunoglobulin (16, 87), or excess free heavy chain toxicity (78). In any case, Raison et al. (113) have demonstrated that mouse-human hybridomas, which ceased antibody production, could be restimulated with lipopolysaccharide (LPS). Similarly, Gronowicz et al. (46) have induced IgM secretion in a murine B-cell tumor with LPS.

Hybridoma cell lines produced in this study secreted McAb of the IgM or IgG classes, with a majority secreting IgM (Table 1). This result was unexpected, as an extended immunization procedure was utilized; therefore, the mouse should have been well within the secondary immune response when sacrificed. The secondary immune response is typified by high IgG titer and low IgM titer.

Cross-reactivity with the heterologous virus was assessed for a majority of the cell lines (Table 1). Most of the cell lines developed using MDMV-A as the immunogen did not cross-react with the heterologous virus MDMV-B.

However, two of the cross-reacting cell lines reacted more strongly with the heterologous virus than with MDMV-A. This suggests that these particular epitopes are represented on MDMV-B in greater numbers or are present in an environment more favorable for antibody binding.

Cell lines developed using MDMV-B as the immunogen were selected, during initial screening procedures, for non-crossreacting antibody secreters. However, of the original 260 MDMV-B positive hybridomas only 18 (7 %) did not cross-react with the heterologous virus MDMV-A. These results suggest that MDMV-B has fewer unique epitopes than does MDMV-A. These results also correlate with the polyclonal studies of Jarjees and Uyemoto (64). They reported polyclonal antisera raised against MDMV-B cross-reacted strongly with MDMV-A, whereas polyclonal antisera raised against MDMV-A cross-reacted only slightly with MDMV-B.

MDMV-A and MDMV-B specific cell lines were further characterized by immunoblot analysis of viral coat protein to identify different epitope specificities exhibited by the McAbs. The assumption is that different immunoblot protein binding patterns produced by the McAbs indicates that the McAbs are epitypically distinct. Based upon this assumption, three different epitopes were identified for each virus strain using these procedures (Figure 2 and

Table 2). Two MDMV-A specific cell lines (MAMIX and MAMXVII) did not bind any viral polypeptides; rather, they produced binding patterns identical to the CM negative control. This result may be attributed to several factors: 1) the concentration of McAb produced in culture may be below the detection sensitivity of the immunoblot procedure, 2) the affinity of the McAbs may be so low that the antibody is desorbed during subsequent incubation and wash steps, or 3) the McAbs may be restricted to structural epitopes which are destroyed during viral protein proteolysis and SDS-PAGE.

Protein bands were also identified with the CM negative control. However subsequent experiments indicated that these bands could be observed on a majority of the immunoblots and were found to correspond to V8 protease bands. It is unlikely that specific recognition of the V8 protease bands occurred. Rather, the V8 protease may inhibit proper blocking of available protein binding sites on the nitrocellulose membrane.

To develop each of the MDMV ds-ELISAs, it was necessary to obtain two epitypically distinct cell lines (50), with one secreting IgM and the other secreting IgG. Several possible combinations for each MDMV strain existed (Table 2). For the MDMV-A ds-ELISA, MAGII was an obvious choice as it was the only MDMV-A specific IgG producing

cell line analyzed by immunoblot. MAMXVIII was utilized as the epitypically distinct IgM producing cell line as the lack of viral polypeptide binding exhibited by MAMIX and MAMXVII was not fully understood. For development of the MDMV-B ds-ELISA, MBGI was utilized as the IgG secreting cell line because it was the only MDMV-B specific cell line which secreted the IgG subclass 2b (Table 1). The IgG 2b subclass has been shown to produce excellent results when purified by protein A sepharose (43). MBMIII was utilized as the epitypically distinct IgM producing cell line as the immunoblot produced by MBMIII was more significantly different from MBGI than those produced by MBMII and MBMIV.

To avoid ELISA background problems associated with contaminating proteins, it was necessary to purify the Ig class from ascitic fluid produced by each respective cell line. The purification of IgG from ascitic fluid using a protein A sepharose column is a well established and rapid procedure (43). The purification of MAGII McAb resulted in excellent yields as indicated by the large IgG peak depicted in Figure 4a. Purification of MBGI, on the other hand, yielded an extremely small IgG peak of low protein content (Figure 4b and Table 6), which was proceeded by a smaller peak not containing specific IgG activity. Results such as this were commonly observed with the Bio-

Rad protein A system and did not seem to be subclass related.

Unlike IgG, a satisfactory procedure for the purification of IgM has not existed (43). Ammonium sulfate precipitation followed by gel filtration has been suggested (43). However, ammonium sulfate also precipitates IgG and a low level of IgG polymers could be detected in the IgM gel filtration peak (results not presented). Contaminating mouse IgG in the IgM preparation will bind to wells of the microtitration plate during IgM coating procedures. Therefore, when rabbit anti-mouse IgG conjugated antibody was added in subsequent steps, bound mouse IgG would be detected in the absence of virus. Thus resulting in high background absorbances or false positives.

To alleviate this problem, IgM was specifically concentrated by precipitation with a 2 % boric acid solution. Boric acid effectively precipitated IgM because of it's low ionic strength, low pH of the boric acid:ascitic fluid mixture, and the propensity of boric acid to form insoluble complexes with carbohydrates (39). The IgM molecule consists of approximately 12 % carbohydrate as compared to IgG which contains only 2 % (102). Boric acid precipitation of IgM, followed by sepharose CL-6B gel filtration, resulted in a McAb

preparation free of mouse IgG.

Sepharose CL-6B gel filtration generated three detectable peaks at fractions 10, 25-30, and 45-50. These peaks are represented by the solid lines in Figures 3a and 3b. The greatest amount of specific, biologically active IgM was obtained in the first peak (broken lines in Figures 3a and 3b). The second and third peaks may represent biologically active, dissociated forms of the IgM pentamer.

When each McAb was obtained in purified form, the ELISA systems were optimized. Optimal concentrations of purified monoclonal IgM, purified monoclonal IgG, and rabbit anti-mouse IgG enzyme conjugated antibody were determined by calculating and plotting the P to N ratios for each antibody preparation. The optimal concentrations used in each system were comparable as depicted in Figures 5, 6, and 7.

Once optimized, the detection sensitivity of each ds-ELISA was assessed using purified virus and virus infected corn tissue. The detection sensitivity of the MDMV-B system using purified virus was 32 ng/ml or 1600 pg/well (Figure 9). The sensitivity of the MDMV-A ds-ELISA was between 16 and 8 ng/ml or 800 and 400 pg/well (Figure 8). However, when MDMV-A infected corn plants were prepared in PBS, no virus was detected by the MDMV-A ds-ELISA. As

indicated above, the assay was extremely sensitive; thus, it was thought that epitopes on the virus in plant samples were not presented to the IgM capture antibody or the IgG second antibody in a recognizable form. Therefore, a high pH carbonate buffer was investigated as a possible grinding buffer in an attempt to expose putative cryptotopes which may be recognized by the capture and second antibodies. As expected, the MDMV-A ds-ELISA specifically detected virus when MDMV-A infected corn tissue was prepared in carbonate buffer (Table 7). Furthermore, when 2.0 % BSA was added to carbonate buffer, the mean A_{410} of MDMV-A infected samples nearly tripled in addition to a complete elimination of background absorbances. The decrease in background was expected; however, the increased specific absorbance was somewhat surprising and still unexplained. The same result, although not as dramatic, was observed when BSA was added to grinding buffer used for MDMV-B infected sample preparations (Table 8). PBS-T at pH 7.2 could be used as the grinding buffer to detect MDMV-B in leaf samples. This suggests that the MDMV-B system recognizes native, intact virions.

Using the appropriate grinding buffer as a diluent, the detection sensitivity of each ds-ELISA was assessed using dilutions of virus infected plant tissue. The MDMV-B system proved to be extremely sensitive as virus was

detected at sap dilutions between 1:2560 and 1:5120 (Figure 10). The MDMV-A system on the other hand, could only detect virus at sap dilutions up to 1:40 (Figure 11). This result was unexpected, as the MDMV-A system was previously shown to detect less than 800 pg of purified virus per well (Figure 8). This loss of sensitivity at high sap dilutions was thought to be related to the pH of the diluted plant sap as all dilutions for the MDMV-A system were performed in a high pH carbonate buffer. To further investigate the effect of pH on the MDMV-A ds-ELISA, MDMV-A infected sap was diluted to 1:20 and adjusted to various pH values between 6.5 and 11.0 before application to the microtitration plate. This experiment demonstrated that the MDMV-A system has a strict pH requirement and can only detect virus in sap at a pH between 7.5 and 8.5 (Figure 12). These results explain the lack of sensitivity demonstrated by the MDMV-A system at high sap dilutions. The pH of dilutions greater than 1:40 were above 9.0 and thus not within the optimal range of the MDMV-A system. These results and the results presented previously in Table 7, indicate that a pH greater than 7.5 was necessary to liberate the cryptotope; however, at a pH greater than 8.5 the epitope is somehow altered and thus undetected by the MDMV-A system.

In summary, the MDMV-A ds-ELISA was performed under

the following conditions: 1) the optimal dilutions of coating IgM (MAMXVIII), second antibody (MAGII), and enzyme conjugated antibody were 1:800, 1:200, and 1:2000, respectively, and 2) samples were prepared in 0.05M carbonate buffer, pH 9.6, containing 2.0 % BSA, at a dilution no greater than 1:40. The MDMV-B ds-ELISA was performed under these conditions: 1) The optimal dilutions of coating IgM (MBMIII), second antibody (MBGI), and enzyme conjugated antibody were 1:1600, 1:400, and 1:2000, respectively, and 2) samples were prepared in 0.01M PBS, pH 7.2, containing 0.05 % Tween 20 and 2.0 % BSA.

The specificity of each fully defined and optimized ds-ELISA was assessed using tissue infected with various MDMV and SCMV isolates. Each system accurately detected virus in samples infected with the homologous virus. The MDMV-B ds-ELISA could detect virus in 18 of 19 (95 %) samples infected with MDMV-B and the MDMV-A ds-ELISA detected virus in 7 of 9 (78 %) samples infected with MDMV-A (Table 9). The three samples not detected by the two assays were prepared by out-of-state cooperators. Therefore, the lack of detection may be a result of microtitration plate shipping and storage, improper sample preparation, or incorrect initial virus strain designation. Furthermore, Jensen's MDMV-A substrains Ohio D, Ohio E, and Ohio F, were originally obtained from

Gingery and correspond to Gingery's MDMV-D, MDMV-E, MDMV-F, respectively. The only difference is that Jensen refers to MDMV-D, MDMV-E, and MDMV-F as MDMV-A substrains Ohio D, Ohio E, and Ohio F, respectively, whereas Gingery recognizes MDMV-D, MDMV-E, and MDMV-F as distinct strains of MDMV. If we were to accept Jensen's strain subdivisions, the MDMV-A ds-ELISA would be 78 % accurate because two of Jensen's MDMV-A substrains obtained from Gingery (MDMV-A Ohio E and MDMV-A Ohio F), did not react in the MDMV-A ds-ELISA. Alternatively, if we accept Gingery's designations, the system recognized MDMV-A and MDMV-F.

In our hands, the assay systems could detect homologous virus with high assurance. The MDMV-A system was found to react with the MDMV-A Ohio D strain supplied by Jensen and cross-react with the MDMV-F strain of Gingery (Table 9). However the MDMV-D strain supplied by Gingery and the MDMV-A Ohio F strain supplied by Jensen did not react in the MDMV-A ds-ELISA as did the MDMV-A Ohio D strain supplied by Jensen and the MDMV-F strain supplied by Gingery. Since MDMV-A Ohio D (Jensen) and MDMV-D (Gingery) as well as MDMV-A Ohio F (Jensen) and MDMV-F (Gingery) are assumed to originate from the same source, a mix-up of cultures supplied by the investigators, or a mutation in the coat protein gene has occurred causing a change in epitope expression.

Louie and Knoke (90) have previously demonstrated the serological relationship between MDMV-A, MDMV-D, and MDMV-F. They also noted that MDMV-A is serologically related to strain E of MDMV; however, cross-reactivity with this strain was not observed in this study. Thus suggesting that the McAbs utilized in the MDMV-A system may be specific for epitopes shared by MDMV-A, MDMV-D, and MDMV-F, which are distinct from common epitopes which may exist between MDMV-A and MDMV strain E. The MDMV-B ds-ELISA was found to strongly cross-react with SCMV-A. The serological relationship between MDMV-B and SCMV-A has also been well established and the strains have been placed in the same serogroup (64, 145).

The goal of this study was to develop a McAb based ds-ELISA which could specifically distinguish the serologically related MDMV strains A and B. Although cross-reactivity with SCMV-A, MDMV-D, and MDMV-F was observed, this cross-reactivity should not diminish the value of the ds-ELISAs for the following reasons. First, maize grown adjacent to sugarcane may become infected with SCMV-A; however, symptom development and crop damage seldom occurs (109). Therefore, plants infected with SCMV-A will most likely be by-passed when collecting field samples. In addition, MDMV-D and MDMV-F are rarely observed in the field and thus would not significantly alter

observed in the field and thus would not significantly alter the effectiveness of the MDMV-A ds-ELISA (141).

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