

**Detection and Differentiation of Maize Dwarf Mosaic Virus, Strains A and B,
by Use of Different Class Immunoglobulins
in a Double-Antibody Sandwich Enzyme-Linked Immunosorbent Assay**

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ABSTRACT

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Murine monoclonal antibodies of two classes and reactive with apparently different epitopes on particles of the A and B strains of maize dwarf mosaic virus (MDMV) were used in a capture indirect enzyme-linked immunosorbent assay (ELISA). The immunoassay used IgM capture and IgG second antibodies. This allowed use of an IgG-specific alkaline phosphatase-conjugated antibody to detect MDMV-A- and MDMV-B-

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bound IgG. Discriminatory capacity of the assay, tested in sap from leaves that were infected with one of several different strains of MDMV and sugarcane mosaic virus (SCMV), was generally sufficient to identify the A and B strains of MDMV, although some cross-reactivity was observed among certain virus strains.

Enzyme-linked immunosorbent assay (ELISA) has been readily adopted for sensitive and specific detection of plant viruses. Many variations have been developed, but, for the detection of plant viruses, most investigators have favored the double-antibody sandwich form of ELISA. In this assay, a capture antibody is employed to coat a solid phase and is used to immobilize the virus. A second antibody, generally conjugated with an enzyme, is used to detect the immobilized virus by virtue of reaction with a substrate appropriate to the enzyme. Numerous studies (e.g., 4,17) have suggested that conjugation of the enzyme to the second antibody can induce conformational changes in the antibody molecule that may alter the avidity and/or affinity of the antibody molecule for the virus and modify strain specificity, which may be undesirable in some circumstances. This problem can often be circumvented by using capture and second antibody prepared in different animal species and detecting the second antibody with an enzyme-conjugated anti-species specific antibody (35). However, specific antisera from different animal species are often unavailable. Methods have been developed that utilize immunoglobulin G (IgG) F(ab')₂ fragments for coating, intact second IgG from the same animal species, and enzyme-conjugated immunoglobulin of protein A Fc-specific reagents (1,18).

The unique characteristics of monoclonal antibodies offer the potential to develop a double-antibody sandwich ELISA that

avoids modification of second antibody reactivity by molecular conjugation and does not require use of antisera raised in different animal species or the preparation of immunoglobulin fragments. Here, we describe such an ELISA, which uses immunoglobulin M (IgM) and IgG monoclonal antibodies as capture and second antibodies, respectively, and commercially available alkaline phosphatase-conjugated anti-mouse IgG to detect antigen-bound IgG. The ELISA depends on use of purified anti-viral IgM, which has no affinity for anti-IgG conjugated antibody. We also describe a new procedure for the purification of IgG-free IgM from ascitic fluid. The sensitivity and discriminating capacity of the assay has been evaluated by assessing reactivity with several different strains of maize dwarf mosaic virus (MDMV) and sugarcane mosaic virus (SCMV). Although previous investigations have distinguished strains A and B of MDMV through use of differential hosts and serology (13,29,32), this report is the first application of a monoclonal antibody-based ELISA for the differentiation of these two plant virus strains.

MATERIALS AND METHODS

Virus origin and purification. Viral immunogens in this study have been described previously as the A (2) and B (ATCC PV53, 10) strains of MDMV.

MDMV-A and MDMV-B were purified from infected *Sorghum sudanese* Piper (Stapf) 'Trudan 5' (Northrup King Co., Minneapolis, MN) and *Zea mays* L. 'Golden Bantam,'

respectively, by a modification (Zeyen and Berger, *personal communication*) of the procedure of Langenberg (21). Infected tissue was homogenized in 0.1 M ammonium citrate adjusted to pH 6.0 with KOH (buffer A) containing 1% polyvinylpyrrolidone (mol. wt. 40,000) and 0.5% 2-mercaptoethanol. Blended tissue was filtered through cheesecloth, and carbon tetrachloride was added to the filtrate to a final concentration of 5%. The preparation was emulsified for 5 sec and centrifuged at 16,000 g for 10 min. Triton X-100 and polyethylene glycol (PEG, mol. wt. 6,000) were added to the supernatant liquid to final concentrations of 0.25 and 6%, respectively. After 30 min, the solution was centrifuged at 22,370 g for 20 min, and the resulting pellet was dispersed in buffer A containing 0.07% 2-mercaptoethanol. The homogenate was centrifuged at 7,700 g for 10 min, and the supernatant was centrifuged through a sucrose pad (8 ml of 20% sucrose in buffer A) for 2 hr at 65,900 g in a Beckman 30 rotor. The pellet was resuspended in buffer A, layered on a 10–40% sucrose gradient, and centrifuged at 96,300 g for 2 hr. Virus particles were collected from a single band in the gradient. An extinction coefficient of $2.4 \text{ (mg/ml)}^{-1} \text{ cm}^{-2}$ at 260 nm (27) was used to estimate virus concentration.

Production of monoclonal antibodies. Female BALB/c mice were immunized with 50 μg of purified virus, emulsified with Freund's complete adjuvant, by the intraperitoneal (IP) route, followed 5–8 wk later by an additional IP injection of 25 μg . Approximately 2 wk after the second injection, 25 μg of virus diluted in 0.1 M sodium phosphate, pH 7.2, containing 0.85% NaCl (PBS) was administered intravenously. Three days later, the mice were exsanguinated, and the serum was retained as a positive control for future testing.

Spleen cells were fused at a 2:1 ratio with nonsecretor SP2/0 Ag-14 cells by modifications of previously described methods (34). Significant modifications included decreasing to 15 sec the time of direct cell exposure to the fusogen (PEG, mol. wt. 1,000, Hazelton-Dutchland, Inc., Denver, CO), increasing the pH of the fusion medium used to dilute cells after fusion from 7.2 to 8.0, and using a rapid limiting-dilution cloning procedure that foregoes cell culture scale-up before cloning (24).

Cells were plated and cultured as described (5). Hybridomas were assayed for specific antibody production by indirect ELISA no later than 10 days post fusion. The contents of all positive wells were cloned repeatedly until all wells containing single colonies were positive. The process of weaning hybridomas off HAT medium was performed during the cloning and expansion procedure. The cell population was increased and frozen, and ascitic fluid was produced as previously described (4).

Antibody class, subclass, and light chain component of each monoclonal antibody were determined by ELISA using each of the following class- and subclass-specific rabbit anti-mouse immunoglobulins; rabbit anti-mouse IgA, IgG1, IgG2a, IgG2b, IgG3, IgM, kappa light chain, and lambda light chain (Zymed Laboratories, Burlingame, CA).

Immunoblot analysis of monoclonal antibodies. Purified MDMV (1 mg/ml) was disrupted with sodium dodecyl sulphate (SDS) and mercaptoethanol (25). Coat protein subunits were partially digested with *Staphylococcus aureus* V8 protease (final concentration 10 $\mu\text{g/ml}$, Sigma Chemical Co., St. Louis, MO) for 4 hr at 37 C (25), electrophoretically separated (19), transferred to nitrocellulose, and probed with monoclonal antibodies to distinguish the monoclonal antibodies on the basis of epitope specificity.

Electrophoretic transfer from the gel to type HAHY 0.45 μm nitrocellulose (Millipore Corp., Bedford, MA) was for 18 hr at 100 mA in 25 mM Tris-Cl, pH 8.3, 192 mM glycine, and 20% methanol; then, nitrocellulose was cut lengthwise into 1.0-cm-wide strips and either stained for total protein with 0.1% naphthol blue black-45% methanol-10% acetic acid and destained with 90% methanol-2% acetic acid or probed with specific monoclonal antibody.

Nitrocellulose strips to be probed with monoclonal antibody were blocked with 3% bovine serum albumin (BSA) in 0.05 M sodium carbonate, pH 9.6, for 2–3 hr at 20 C. After three washes with 0.05 M Tris-Cl, pH 7.4, containing 0.05% Tween-20 (wash

buffer), each strip was incubated overnight at 20 C in culture medium (diluted 1:5 in wash buffer) containing specific monoclonal antibody. After being washed again, nitrocellulose strips were incubated for 1 hr with a mixture of alkaline phosphatase-conjugated rabbit-anti-mouse IgG and goat-anti-mouse IgM (Sigma), both diluted 1:1,000 in wash buffer containing 1% BSA. After additional washing in wash buffer, strips were rinsed three times (3–5 min each) with 0.1 M Tris-Cl, pH 7.5, 0.1 M NaCl, 2.0 mM MgCl_2 containing 0.05% Triton X-100 and then with three changes, over a 30-min period, of 0.1 M Tris-Cl, pH 9.5, 0.1 M NaCl, 5.0 mM MgCl_2 . A mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium, prepared according to Leary et al (22), was added, and 15–20 min later, the reaction was terminated by addition of 0.01 M Tris-Cl, pH 7.5, containing 1 mM EDTA.

Isolation of monoclonal antibodies. Monoclonal IgG was isolated by affinity chromatography of ascitic fluid on a Bio-Rad Affi-gel protein A MAPS column (Bio-Rad Laboratories, Inc., Richmond, CA) by using procedures and buffers provided by the manufacturer. Eluted IgG fractions were detected spectrophotometrically at $A_{280\text{nm}}$ and collected in 1.0-ml aliquots into a tube containing 2.0 ml of 0.1 M Tris-Cl, pH 9.0.

Monoclonal IgM was purified from ascitic fluid by dropwise addition of ascitic fluid to 0.32 M boric acid with gentle stirring (ascitic fluid:boric acid ratio, 1:20). After incubation of the mixture at 20 C for 30 min, the precipitated antibody was collected by centrifugation at 800 g for 10 min. The antibody was resuspended in 0.1 M Tris-Cl, pH 8.0, containing 0.15 M NaCl and dialyzed against the same buffer overnight. The mixture was chromatographed on a Sepharose CL-6B (Pharmacia, Inc., Piscataway, NJ) gel filtration column (58 cm \times 1.5 cm, $V_0 = 35$ ml) by using 0.1 M Tris-Cl, pH 8.0, containing 0.15 M NaCl as the elution buffer. One-milliliter fractions after the void volume were collected and assayed by indirect ELISA for presence and absence of specific IgM and IgG, respectively. An extinction coefficient of $1.4 \text{ (mg/ml)}^{-1} \text{ cm}^{-2}$ was used to estimate protein concentration of monoclonal antibody.

Indirect ELISA. The indirect ELISA used for detection of specific antibody was similar to that described by Voller et al (36). Purified virus antigen (5 $\mu\text{g/ml}$) in 50 μl of PBS was added to wells of Immuno I (Vangard International, Neptune, NJ) microtitration plates. After incubation, plates were washed with 0.01 M sodium phosphate, pH 7.2, containing 0.15 M NaCl and 0.05% Tween 20 (wash buffer), and unbound protein-binding sites were blocked with BLOTTO (15) prepared in PBS. After incubation, plates were rinsed with wash buffer. Specific antibody was bound to immobilized MDMV by adding 50 $\mu\text{l/well}$ of hybridoma culture medium. Controls included hyperimmune polyclonal mouse serum (1:1,000 in PBS), normal mouse serum (1:1,000), and conditioned medium (CM as described by Diaco et al [4]). Alkaline phosphatase-conjugated rabbit anti-mouse IgG or anti-mouse IgM (Sigma) was added (50 $\mu\text{l/well}$, diluted 1:1,000 in wash buffer containing 1% BSA [Sigma]) independently or in a mixture. The plate was incubated and washed as described. All incubations were performed in a humidified chamber for 1 hr at 20 C or overnight at 4 C. Substrate (50 $\mu\text{l/well}$, 1 mg/ml *p*-nitrophenylphosphate in 10% diethanolamine, pH 9.8) was added, and, after 30–60 min, the enzyme reaction was terminated by addition of 3 N NaOH (50 $\mu\text{l/well}$). Substrate conversion was measured at $A_{410\text{nm}}$ in a Dynatech Minireader II (Dynatech Laboratories, Inc., Alexandria, VA).

Double-antibody sandwich ELISA. The double-antibody sandwich ELISA used immunoglobulins of different classes. IgM capture and IgG second antibodies specific for distinct virus epitopes were diluted to optimal concentrations as determined by calculation of P/N ratios (9). After addition of the IgM capture antibody (50 $\mu\text{l/well}$), diluted in 0.05 M carbonate-bicarbonate buffer, pH 9.6, to wells of microtitration plates and subsequent washing, the procedures were identical to those described in the indirect ELISA except that only alkaline phosphatase-conjugated rabbit anti-mouse IgG, diluted 1:2,000 as determined by calculation of the P/N ratio, was used as the enzyme-conjugated

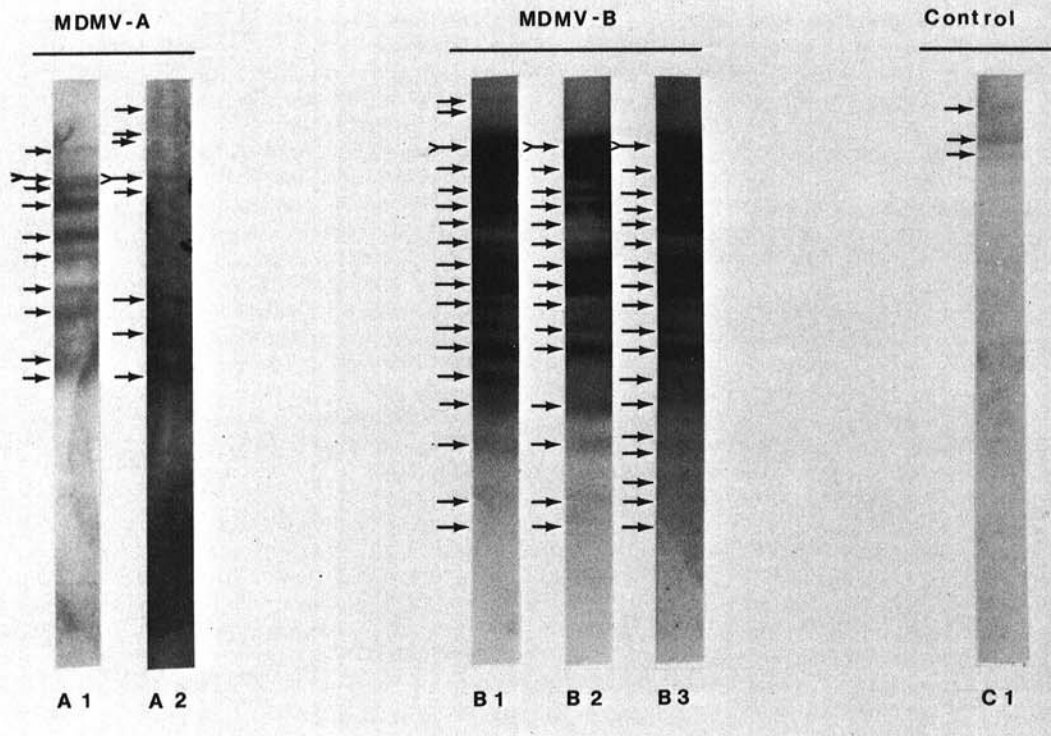


Fig. 1. Immunoblot analysis of MDMV-A, MDMV-B specific cell lines, and CM control (C1). Viral protein was digested with V8 protease, polypeptides were electrophoretically separated on SDS slab gels (14 cm × 16 cm × 1.5 mm) using stacking and resolving gels of 4 and 15% polyacrylamide, respectively, transferred to nitrocellulose and probed with specific monoclonal antibodies. A1-A2, B1-B3, and C1 differentiate the patterns observed. Undigested coat protein is represented by \blacktriangleright .

TABLE 1. Cell line designation, monoclonal antibody subclass, light chain secreted, and immunoblot binding pattern exhibited by hybridomas produced to MDMV-A and MDMV-B

Cell line ^a	IgG Subclass	Immunoblot pattern ^c
MAGII	IgG2aK	A1
MAMIV	... ^b	A1
MAMV	...	A1
MAMVII	...	A1
MAMIX	...	C1
MAMXII	...	A1
MAMXIV	...	A1
MAMXVII	...	C1
MAMXVIII	...	A2
MAMXIX	...	A1
MBG1	IgG2bK	B1
MBGII	IgG2aK	B1
MBGIII	IgG1k	B1
MBGIV	IgG2aK	B1
MBGV	IgG1K	B1
MBGVI	IgG3K	B3
MBMI	...	B1
MBMII	...	B3
MBMIII	...	B2
MBMIV	...	B3
MBMV	...	B1

^a Cell lines are designated by the original immunogen used to produce and screen the line (MDMV-A = MA, MDMV-B = MB), the antibody class secreted by the cell line (IgG = G, IgM = M) and a roman numeral to distinguish cell lines that have the same three-letter designation.

^b Not applicable to this cell line.

^c Patterns refer to those illustrated in Fig. 1.

antibody. Leaf tissue samples were prepared by grinding approximately 0.5 g of infected tissue in 4.5 ml of an appropriate buffer with a mortar and pestle. Samples were considered positive if the A_{410nm} was greater than 0.10.

RESULTS

Preparation and characterization of monoclonal antibodies. A total of 1,001 wells, of all those tested, contained hybridomas from two separate fusions using MDMV-A or MDMV-B as immunogens. Specific antibody was secreted by 746 of these hybridomas. Originally, 260 hybridomas reacted positively with MDMV-B. Eighteen of these did not cross react with MDMV-A in indirect ELISA and were cloned immediately to yield 11 hybridomas that secreted MDMV-B specific antibody. Six of these monoclonal antibodies were of the IgG class, and five were IgM.

Fifty hybridomas reactive to MDMV-A were chosen for cloning, and 26 of these were successfully established. Twenty-three of these MDMV-A hybridomas secreted monoclonal antibodies of the IgM class. Six of these were cross reactive with MDMV-B, 10 reacted only with MDMV-A in indirect ELISA, and seven were not tested for cross reactivity. The remaining three monoclonal antibodies were of the IgG class; one was cross reactive with MDMV-B in indirect ELISA, one reacted only with MDMV-A, and the third was not tested for cross reactivity.

Efficiency of hybridoma production was directly related to the specific serum titer of the immunized mouse. The immunization procedure of Hill et al (8) resulted in serum titers between 1:256 and 1:512, and the subsequent spleen cell fusion yielded few hybridomas (2–10% of the cultured wells). The immunization procedure described in this study resulted in serum titers of 1:12,000 or greater, and successful hybridomas were obtained in 50–70% of the cultured wells. Furthermore, less than 1% of the successful hybridomas obtained from the mouse with low serum titer secreted immunogen-specific antibody, whereas 75% of the successful hybridomas obtained from the spleen of the mouse with high serum titer secreted specific antibody.

Immunoblot analysis of monoclonal antibodies. Cloned hybrids producing monoclonal antibodies of either class that did not cross react with the heterologous strain were chosen for use in immunoblot experiments to probe V8 protease digests of homologous viral coat protein. Binding of monoclonal antibodies

to polypeptides resulting from partial digestion of coat protein revealed six different patterns and reflected apparent epitope specificity of the different antibodies. Three patterns were produced by the MDMV-A specific cell lines, three were produced by the MDMV-B specific cell lines, and the CM negative control also produced a pattern identical to that produced by two MDMV-A specific cell lines (Fig. 1, Table 1). Nitrocellulose, stained with naphthol blue black, revealed only some of the polypeptides detected by probing nitrocellulose with monoclonal antibodies (data not shown). This is characteristic of the relative difference in protein detection sensitivity by staining as compared to probing of nitrocellulose with antibodies and has been frequently observed with other viral proteins in our laboratory (*unpublished*). Further experiments with molecular weight standards suggested that all three bands evident in the immunoblot probed with CM had higher molecular weights than the viral protein subunit and therefore were not of viral origin. Furthermore, they were also detected when a control, containing only V8 protease, was probed. The bands were also evident when antibodies from a majority of the cell lines were used in the immunoblots employing MDMV-A and MDMV-B as antigens and are identified as the upper band of A1, upper three bands of A2, and upper two bands of B1 (Fig. 1). It is possible that the enzyme inhibits blocking of the nitrocellulose rather than presenting recognition sites for monoclonal antibodies. Alternatively, because V8 is a protease, it may retain protein binding sites even after electrophoresis in SDS gels and transfer to nitrocellulose.

Purification of mouse IgM. Monoclonal mouse IgM purified by borate precipitation and Sepharose gel filtration (Fig. 2) showed no reactivity to anti-mouse IgG. Ten milliliters of ascitic fluid yielded approximately 6.5 mg of protein.

Strain-specific double-antibody sandwich ELISA. Monoclonal antibodies specific for distinct viral epitopes were chosen for detection of MDMV. One cell line secreted IgM and the other secreted IgG. For the MDMV-A double-antibody sandwich ELISA, cell line MAGII was used because it was the only MDMV-A-specific IgG-producing cell line analyzed by immunoblot. The apparent lack of viral polypeptide binding to monoclonal antibodies produced by cell lines MAMIX and MAMXVII was not fully understood and, therefore, cell line MAMXVIII was used as the producer of IgM because it was the only other IgM that did not react with the A1 pattern. For development of the MDMV-B

double-antibody sandwich ELISA, MBGI was used as the IgG-secreting cell line because it was the only MDMV-B-specific cell line that secreted the IgG subclass 2b (Table 1). The IgG2b subclass has been shown to produce excellent results when purified by protein A Sepharose (7). The cell line MBMIII was used as the IgM-producing cell line because its immunoblot was more easily distinguished from MBGI than the pattern produced by MBMII and MBMIV. Optimal protein concentrations of purified antibody from cell lines MAMXVIII, MAGII, MBMIII, and MBGI, as determined by calculation of P/N, were 0.70, 7.45, 0.26, and 1.07 $\mu\text{g/ml}$, respectively. Alkaline phosphatase-conjugated rabbit anti-mouse IgG was used at a concentration of 0.45 $\mu\text{g/ml}$.

The limit of detection of the MDMV-specific double-antibody sandwich ELISA, as determined by using purified virus diluted from 1.0 to 1,000 ng/ml, was 16 ng/ml (0.8 ng/well) and 32 ng/ml (1.6 ng/well) for the A and B strains, respectively (data not shown). No cross reactivity was observed with purified heterologous virus ranging in concentration from 10 to 10,000 ng/ml.

Detection of virus in infected maize leaf samples depended on the buffer used. Results of experiments in which homogenates of infected leaves were prepared in PBS-Tween or 0.05 M carbonate-bicarbonate buffer, pH 9.6, with and without 2% BSA, showed that the carbonate buffer containing BSA gave the greatest amount of reaction product, as measured by $A_{410\text{nm}}$ when MDMV-A was used as the antigen and that PBS-Tween containing BSA was best for MDMV-B (Table 2).

The detection level for each strain-specific ELISA was assessed by grinding infected maize leaf tissue with a mortar and pestle in PBS-Tween containing BSA or carbonate buffer containing BSA for the B and A strains of MDMV, respectively, and preparing dilutions in the same buffer ranging from 1:10 to 1:5,120. Results showed that the MDMV-B ELISA specifically detected viral antigen in sap at a dilution end point of 1:2,560, whereas the MDMV-A-specific ELISA detected virus in sap at dilutions only up to 1:40 (Fig. 3). Because serial dilution of MDMV-A-infected samples was in carbonate buffer, the pH of the sample increased with sample dilution. Therefore, the pH dependency of MDMV-A detection was examined.

Maize leaf tissue infected with MDMV-A was ground in PBS at a tissue to buffer ratio of 1:20, and samples, adjusted to pH values, at intervals of 0.5, from 6.5 to 11.0, were assayed by double-antibody sandwich ELISA. Results indicated that efficacy of the

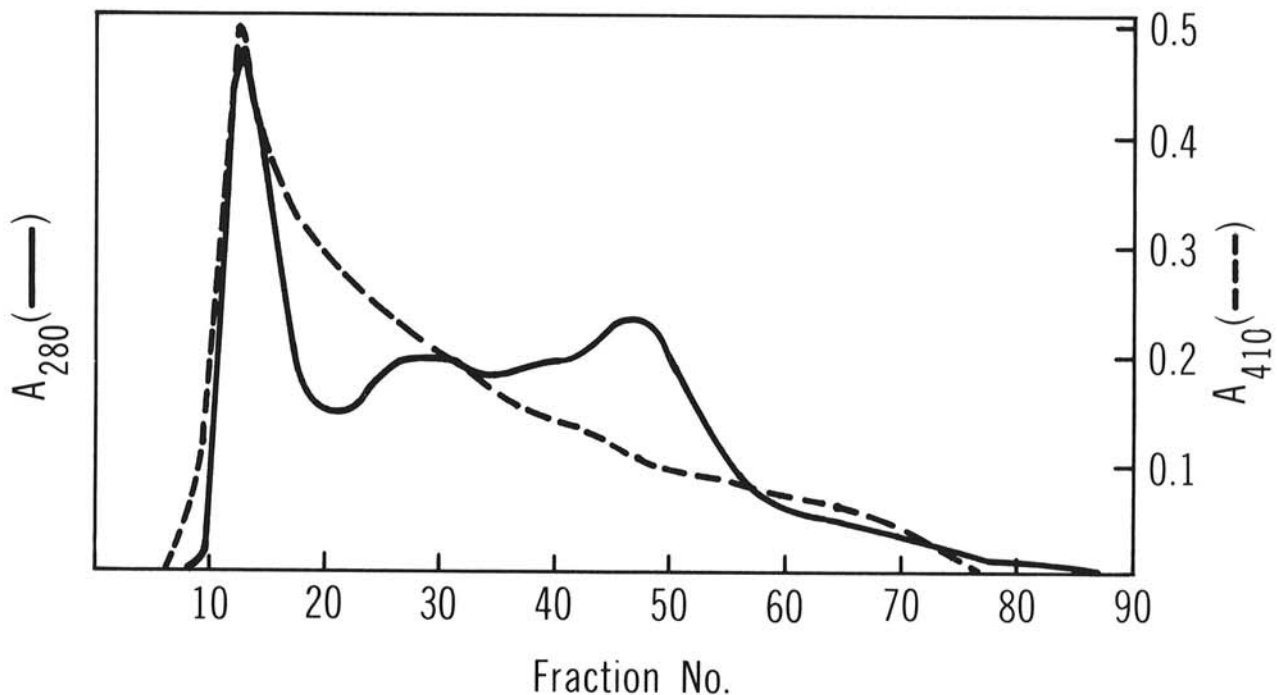


Fig. 2. Elution profile (o—o) and antibody activity (---) of MDMV-A specific IgM monoclonal antibody purified through Sepharose CL-6B. Monoclonal antibody activity was determined by indirect ELISA.

TABLE 2. Reaction of maize dwarf mosaic virus (MDMV) infected and healthy corn leaf tissue prepared in various buffers with a strain-specific, monoclonal antibody class-based indirect, double-antibody sandwich ELISA

Sample	Monoclonal antibody specificity	Grinding buffer ^a			
		PBS-Tween	PBS-Tween-BSA	Carbonate	Carbonate-BSA
Buffer control	MDMV-A	0.000 ^b	0.000	0.000	0.000
Healthy leaf tissue	MDMV-A	0.005	0.005	0.003	0.000
MDMV-A infected leaf tissue	MDMV-A	0.015	0.008	0.140	0.405
MDMV-B infected leaf tissue	MDMV-A	0.008	0.000	0.000	0.000
Buffer control	MDMV-B	0.000	0.000	0.000	0.000
Healthy leaf tissue	MDMV-B	0.005	0.000	0.000	0.000
MDMV-A infected leaf tissue	MDMV-B	0.000	0.003	0.000	0.005
MDMV-B infected leaf tissue	MDMV-B	0.165	0.238	0.005	0.005

^a Grinding buffers were 0.01 M sodium phosphate, pH 7.2, containing 0.05% Tween 20 (PBS-Tween), PBS-Tween containing 2% bovine serum albumin (BSA), 0.05 M sodium carbonate, pH 9.6 (carbonate), and carbonate containing 2% BSA.

^b Data are the mean A_{410nm} values of four identical samples. Values greater than 0.100 were considered positive.

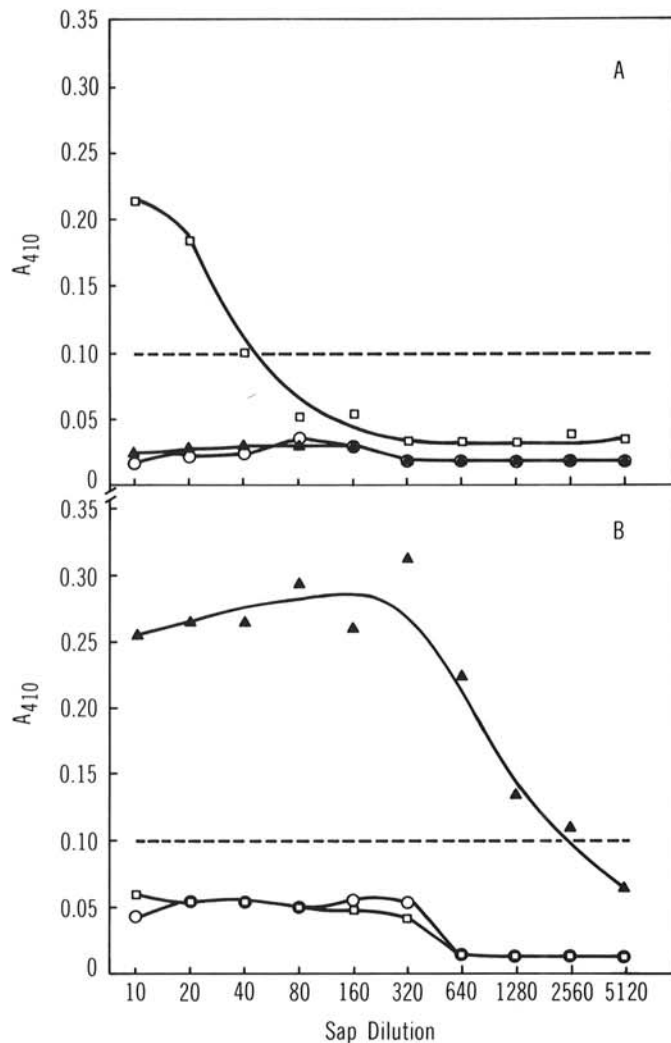


Fig. 3. Detection sensitivity of A, MDMV-A and B, MDMV-B isotype-specific double-sandwich ELISA in sap from infected leaf tissue. MDMV-A-infected (□—□), MDMV-B-infected (▲—▲) and uninfected (○—○) maize leaf tissue were ground in (A) 0.05 M carbonate buffer, pH 9.6, containing 2% BSA or (B) PBS-Tween containing 2% BSA. Dilutions of sap were made in the same buffers used for tissue grinding. Data points are the mean values of duplicate experiments. Values greater than A_{410} of 0.10 were considered positive.

MDMV-A-specific ELISA was pH dependent, with the optimal sample pH occurring between 7.5 and 8.5. Dilution experiments, similar to those described except that all dilutions were adjusted to pH 7.5–8.5, revealed that MDMV-A could now be detected in sap at a dilution of 1:160.

Several microtitration plates coated with specific IgM capture antibody and blocked with BLOTTO were shipped via express mail to R. E. Gingery (Wooster, OH) and S. G. Jensen (Lincoln, NE). The cooperators applied virus-infected maize samples of known strain designations to the plates by using appropriate buffers. After incubation and washing, the plates were returned and analyzed in our laboratory. In addition, microtitration plates were also used for analysis of several local MDMV isolates that had been identified by phenotypic response on differential host plants (*unpublished*). Results revealed that 13 locally collected isolates of MDMV-B reacted specifically in the MDMV-B double-antibody sandwich ELISA and not in the MDMV-A specific ELISA. Five isolates each of MDMV-A and MDMV-B applied to the plates by one cooperator reacted specifically with the assays and showed no cross reactivity with antibody to the heterologous virus. One isolate, identified as MDMV-B, did not react in either assay. Neither assay detected MDMV strains E or O from Gingery, E or KS1 from Jensen, or SCMV strains B, H, or M (14). SCMV-A cross reacted strongly with the MDMV-B ELISA but did not react with the MDMV-A assay. In contrast to these results, MDMV-D provided by Jensen was reactive in the MDMV-A specific ELISA but, when provided by Gingery, did not react in either the MDMV-A or MDMV-B ELISA. Similarly, MDMV-F provided by Jensen was unreactive in both assays, but, when provided by Gingery, it reacted with the MDMV-A-specific ELISA. Control plates, including those coated with specific IgM, shipped to cooperators and returned to our laboratory for application of antigen, and also those coated with IgM and antigen, shipped and returned, suggested that the reagents were stable in mail transit.

DISCUSSION

Enhanced production of relevant hybridomas was achieved by using mice with high serum titers, reducing the time of direct cellular exposure to PEG to protect newly formed hybridomas from the cytotoxic effects of PEG (20), and increasing the fusion medium pH from 7.2 to 8.0 (16,28,37). Most of the cell lines developed by using MDMV-A as the immunogen did not cross react with the heterologous MDMV-B. In contrast, only 7% of the 260 MDMV-B positive hybridomas did not cross react with the heterologous MDMV-A. These results suggest that MDMV-B has fewer unique epitopes than does MDMV-A and are in agreement with the studies of Jarjees and Uyemoto (13), who observed that polyclonal antisera raised against MDMV-B cross reacted strongly with MDMV-A, but that antisera raised to MDMV-A cross reacted only slightly with MDMV-B.

To avoid labeling the second antibody, the design of the double-antibody sandwich ELISA was based on an IgM capture antibody and an IgG second antibody. IgM was chosen as the capture antibody because of its greater avidity for specific antigen and its greater affinity towards plastics than IgG (30).

A satisfactory purification procedure for IgG-free IgM from ascitic fluid has not been reported. Ammonium sulfate precipitation followed by gel filtration (7) fails to remove all traces

of IgG. IgG is precipitated by ammonium sulfate, and a low level of contaminating polymeric IgG can be detected in the eluted IgM fraction (*unpublished results*). Therefore, the higher carbohydrate content of IgM as compared with IgG and the propensity of boric acid to form insoluble complexes with carbohydrates (6) was used for purification of IgM.

Previous investigation using the monoclonal antibodies S1 and S2 prepared to soybean mosaic virus suggested that maximum sensitivity of a double-antibody sandwich assay using these antibodies was achieved when antibodies were specific to different epitopes (8). Therefore, monoclonal antibodies specific to apparently different epitopes on MDMV were selected for this study. Competition experiments have been used to discern such antibodies (4,8). Such experiments, however, may have certain inherent difficulties. Inhibition may indeed be due to competition for the same epitope. However, the experiments do not always unequivocally differentiate this from inhibition of binding of a second antibody specific for another epitope if the two epitopes are in proximity or overlapping; also, inhibition may be due to steric hindrance caused by binding of the first antibody. In addition, the first antibody may prevent access of a second antibody by altering the conformation of the epitope specific to the second antibody or even the entire antigen (11,12). An alternative approach is to examine the diversity of hybridomas by immunoblotting of peptides produced by Cleveland maps (3) of MDMV coat protein. Three different binding patterns were observed for each strain of MDMV. Two MDMV-A-specific cell lines did not bind any viral polypeptides; rather, they produced binding patterns identical to the CM negative control. Several factors, including low concentration of monoclonal antibody produced in culture, low affinity of the monoclonal antibody or the reactivity of the antibodies to conformational epitopes destroyed during proteolysis and SDS gel electrophoresis, or inability of antibody to react with antigen when nitrocellulose, in contrast to microtiter plates, is used as the solid phase may have contributed to these results.

The indirect double-antibody sandwich ELISA accurately detected virus in samples infected with the homologous virus. The MDMV-B ELISA detected virus in 18 of 19 (95%) samples infected with MDMV-B, and the MDMV-A assay detected virus in five of five (100%) samples infected with MDMV-A. Heterologous reactivity of the MDMV-B-specific ELISA was detected only with SCMV-A. A discrepancy was observed in the cross-reactivity of MDMV strains D and F provided by our two cooperators. Because the MDMV-D and -F strains maintained by S. Jensen originated from R. Gingery but were obtained from a third party (S. Jensen, *personal communication*), these data suggest that either erroneous strain identification or antigenic drift may have occurred within these viral strains. Therefore, MDMV-D and MDMV-F were subsequently directly acquired again from the Ohio cooperators. Simultaneous electrophoretic analyses of viral coat protein from the two collections of MDMV-D and MDMV-F by the technique of Jensen et al (14) revealed no difference from previously reported values and showed no detectable differences among the two collections of each of the virus strains (S. Jensen, *personal communication*). Therefore, it is likely that antigenic drift occurred. This may be the first example of the occurrence of antigenic drift in plant viruses.

Louie and Knoke (23) have previously demonstrated serological relationships among MDMV-A, MDMV-D, and MDMV-F. They also noted that MDMV-A is serologically related to MDMV-E; however, a heterologous reaction with this strain was not observed in this study. The serological relationship between MDMV-B and SCMV-A has been well established, and the strains have been placed in the same serogroup (13,33).

The degree of heterologous reactivity observed among virus isolates used in this study is unlikely to preclude the potential use of monoclonal antibodies for identification of MDMV strains. Although maize grown adjacent to sugarcane may become infected with SCMV-A, symptom development and crop damage seldom occur (26). In addition, MDMV-D and MDMV-F are rarely observed in the field, and this would not significantly alter the

effectiveness of the MDMV-A ELISA (31). We have also demonstrated the feasibility of using different class antibodies in double-sandwich immunoassays, which avoids the necessity for labeling virus-specific antibody and allows the effective use of anti-viral IgM monoclonal antibodies for plant virus identification.

LITERATURE CITED

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