Production of Monoclonal Antibodies to Peanut Mottle Virus and Their Use in Enzyme-Linked Immunosorbent Assay and Dot-Immunobinding Assay

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Journal Article 5089 of the Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater. Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by Oklahoma State University nor imply its approval to the exclusion of other products or vendors that may also be suitable.

Accepted for publication 19 February 1987 (submitted for electronic processing).

ABSTRACT

Sherwood, J. L., Sanborn, M. R., and Keyser, G. C. 1987. Production of monoclonal antibodies to peanut mottle virus and their use in enzyme-linked immunosorbent assay and dot-immunobinding assay. Phytopathology 77:1158-1161.

Stable hybridoma cell lines secreting monoclonal antibodies (MAB) to peanut mottle virus (PMV) were produced by fusing spleen cells of immunized mice and mouse myeloma cell line P3X63Ag8.653. Hybridoma clones produced antibodies of the IgG1 subclass. The MAB reacted to nine isolates of PMV but did not react to eight other viruses tested. The immunoreactivity of the MAB was compared with polyclonal rabbit serum for detection of PMV in foliar tissue by enzyme-linked immunosorbent assay (ELISA) and a dot-immunobinding assay. An ELISA using both polyclonal antibodies and MAB was superior to assays using either source of antibody alone. However, a dot-immunobinding assay using only MAB was satisfactory for detection of PMV.

Additional key words: polyclonal antiserum, potyvirus.

Peanut mottle virus (PMV), a member of the potyvirus group, is capable of infecting a number of legume species (4,5). Although numerous isolates of PMV have been distinguished by biological properties (1,11,14), nucleic acid hybridization studies and studies with polyclonal antiserum show a strong relationship among isolates (1,2). PMV often is difficult to purify because of aggregation of virus particles, which makes the routine production of highly specific polyclonal antiserum difficult. The monoclonal antibody (MAB) technology provides a means to produce a potentially unlimited supply of highly specific uniform antibody (10), which is useful in the detection of plant viruses (6). The purpose of this study was to produce hybridomas secreting PMV-specific MAB, test the reaction of selected MAB to isolates of PMV, and develop an enzyme-linked immunosorbent assay (ELISA) and dot-immunobinding assay for the detection of PMV in foliar tissue. Preliminary reports have been published (20,21).

MATERIALS AND METHODS

Viruses, purification, and polyclonal antiserum production. The PMV isolate (PMV-OK) used for MAB production was maintained in pea (*Pisum sativum* L. 'Little Marvel') and purified as previously described (16,17). Other isolates of PMV obtained from C. W. Kuhn, University of Georgia, had been isolated in Georgia and differentiated based on host reaction, except the PMV-IND isolate from India (Table 1). These isolates also were maintained in Little Marvel pea. Other viruses used in serological assays were maintained in appropriate hosts. Polyclonal antiserum to PMV-OK was produced in rabbits as previously reported (19).

Hybridoma production, screening, and isotyping. The mouse myeloma cell line P3X63Ag8.653 (653) was provided by E. L. Halk (Agrigenetic Corporation, Madison, WI) and maintained at 37 C with 5% CO_2 in RPMI 1640 medium (K. C. Biological, Inc., Lenexa, KS) that was supplemented with 10% horse serum (HyClone Laboratories, Logan, UT), 1.0 g/L of $NaHCO_3$, 0.06 g/L of penicillin-G, 0.10 g/L of streptomycin sulfate, and 0.30 g/L of L-glutamine (Sigma Chemical Co., St. Louis, MO). Suspensions of 653 cells were diluted with an equal volume of fresh medium on each of three days before fusion to insure log phase growth at the time of fusion.

After fusion, cells were placed in SP medium (40% RPMI 1640, 40% conditioned medium, 20% horse serum, and 0.05 mg/ml of gentamicin) supplemented with 10^{-4} M hypoxanthine, 4×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine (HAT). Conditioned medium is supernatant from cultured cells prepared by adding 100 ml of a myeloma cell suspension to 500 ml of RPMI 1640 medium for 24 hr, followed by removal of the cells by centrifugation at 400 *g* for 5 min. The hybridoma cells were switched to HT medium (HAT without aminopterin) after 1 wk and maintained in SP medium after a week in HT medium.

BALB/c mice were immunized by intramuscular injection with 250 μg of PMV-OK emulsified with Freund's complete adjuvant. Three injections were made 1 wk apart, followed by an injection of 250 μg of virus in distilled water 3 days before fusion.

The procedure for cell fusion was adapted from the method of Kohler and Milstein (10). The immunized mice were sacrificed and the spleens aseptically removed. Spleens were rinsed three times in a balanced salt solution (140 mM NaCL, 5 mM KCl, 4 mM $NaCO_3$, 5.6 mM glucose, and 10 ml/L of a 0.2% phenol red solution). The spleens were then gently pressed through 0.1 mm^2 nylon mesh into 10 ml of serum-free RPMI 1640. Cells were centrifuged at 400 *g* for 7 min and the pellet resuspended in 10 ml of serum-free RPMI 1640. An equal number of 653 cells was pelleted under the same conditions and resuspended in 20 ml of serum-free RPMI 1640. The two cell suspensions were mixed and centrifuged as before. Pellets were gently resuspended in 12 ml of 35% PEG-1000 in serum-free RPMI 1640, pH 7.8, and centrifuged at 190 *g* for 2 min. The cells were placed in a 37 C water bath and left undisturbed for 6 min. The PEG was removed by two subsequent centrifugations at 190 *g* for 8 min in 20 and 30 ml of SP medium, respectively. The pellet was resuspended in 40 ml of SP medium and incubated overnight in a 75-cm^2 tissue culture flask. The next day, the cells were centrifuged at 400 *g* and the pellet resuspended in approximately 125 ml of HAT medium and distributed in 24-well tissue culture plates.

Approximately 2 wk after fusion, hybridomas were screened for antibody production by an indirect double sandwich ELISA. After coating plates with rabbit anti-PMV IgG at 1 μg/ml for 2 hr, purified PMV was added to the wells at 3 μg/ml and stored at 4 C overnight. Plates were washed three times and then blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 10 min. Undiluted culture supernatant was added, incubated for

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TABLE 1. Reaction of peanut mottle virus (PMV)-specific monoclonal antibodies to selected PMV isolates in an indirect double sandwich ELISA[a] (A_{405nm})

Sample	MAB from hybridoma clone		
	10D4	10A4	9A1
PMV-OK (16,17)	1.400 ± 0.120[b]	0.558 ± 0.041	1.504 ± 0.122
PMV-LB (from lima bean)	1.176 ± 0.123	0.432 ± 0.037	1.268 ± 0.116
PMV-N (14)	1.263 ± 0.126	0.436 ± 0.022	1.265 ± 0.081
PMV-NC (necrosis in peanut)	1.347 ± 0.130	0.569 ± 0.037	1.584 ± 0.072
PMV-M (mild symptoms in peanut)	1.219 ± 0.095	1.463 ± 0.111	0.595 ± 0.033
PMV-IND (15)	0.490 ± 0.041	0.598 ± 0.072	0.301 ± 0.015
PMV-D (from *Desmodium*)	0.669 ± 0.052	0.786 ± 0.032	0.314 ± 0.013
PMV-AR (from arrowleaf clover)	1.191 ± 0.093	1.343 ± 0.110	1.147 ± 0.072
PMV-CS (11)	0.799 ± 0.054	1.036 ± 0.088	0.520 ± 0.036
Phosphate-buffered saline	0.100 ± 0.010	0.090 ± 0.009	0.095 ± 0.012
Healthy pea	0.105 ± 0.007	0.100 ± 0.006	0.083 ± 0.007

[a] Plates were coated with rabbit polyclonal anti-PMV IgG at 1 μg/ml. Samples were added at a 1:100 dilution. Samples were probed with undiluted culture supernatant followed by alkaline phosphatase labeled goat anti-mouse antibody. Values are averages of three experiments.
[b] ± = Standard error.

2.5 hr, followed by alkaline phosphatase-linked goat anti-mouse IgG at the manufacturer's recommended working dilution (No. A5153, Sigma Chemical Co., St. Louis, MO). After an additional 2.5 hr of incubation the enzyme substrate was added.

Hybridoma cell lines that gave positive reactions were cloned by using the soft agar method. Approximately 50 μl of hybridoma cell suspension was placed in a 10-cm petri dish followed by 10 ml of cloning medium (100 ml of SP medium, 2.4 ml of Gibco amino acids with L-glutamine, 5×10^{-2} M 2-mercaptoethanol, and 6.5 ml of 5% Difco agar in water). This mixture was swirled, and plates were incubated for 2 wk. Single colonies were picked and transferred into 1 ml of SP medium in 24-well plates. The culture supernatants were retested for antibody production, and positive cell cultures were recloned an additional two times.

A kit for identification of subclass specific antisera (Zymed Laboratories, Inc., San Francisco, CA) containing rabbit anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, kappa light chain, and lambda light chain antisera was used for isotyping the MAB.

Ascites production and MAB purification. Ascitic fluid containing MAB was produced in pristane-primed BALB/c mice by injecting approximately 10^6 hybridoma cells into the peritoneal cavity and then collecting ascitic fluid in 2 to 3 wk (7). MAB was obtained from the ascitic fluid using an IgG affinity purification kit from HyClone Laboratories or by precipitation by ammonium sulfate.

ELISA and dot-immunobinding assay. Direct and indirect double sandwich ELISA procedures were modified after that of Clark and Adams (3). Plates were coated with the IgG fraction from rabbit anti-PMV polyclonal serum or from mouse ascitic fluid in 0.05 M carbonate buffer, pH 9.6, for 2 hr at room temperature. Plates were rinsed three times with phosphate-buffered saline containing 0.05% Tween (PBS-Tween), and samples diluted in PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) were then added. After incubation overnight at 4 C, plates were rinsed three times with PBS-Tween. Alkaline phosphatase (No. P5521, Sigma Chemical Co.) labeled anti-PMV IgG from polyclonal serum, alkaline phosphatase labeled anti-PMV IgG from ascitic fluid, or MAB culture supernatant diluted with PBS-Tween containing 2% PVP and 0.2% ovalbumin was added. In the latter case, after a 3-hr incubation the plates were rinsed as above, and alkaline phosphatase labeled goat anti-mouse antibody at the manufacturer's recommended working dilution was added and incubated for an additional 3 hr. The alkaline phosphatase labeled anti-PMV IgGs were incubated for 5 hr. After incubation at room temperature, plates were rinsed as above, the *p*-nitrophenyl phosphate in diethanolamine substrate buffer was added, and plates were incubated before reading in a Bio-Tek EIA plate reader (Bio-Tek Instruments, Inc., Burlington, VT).

To determine the optimal concentration of reagents for use in ELISAs, plates were initially coated with various concentrations of the IgG fraction from polyclonal antiserum or ascitic fluid, and, in the direct double sandwich ELISAs, samples were probed with different dilutions of conjugated antibody. For the indirect double sandwich ELISA, samples were probed with different dilutions of hybridoma culture supernatant. From the results obtained in these experiments, plates were first coated with either 1.0 μg/ml of IgG from polyclonal serum or ascitic fluid, and, in the direct double sandwich ELISAs, samples were probed with the alkaline phosphatase linked IgG from polyclonal antiserum at a 1:800 dilution and the alkaline phosphatase linked IgG from ascitic fluid purified by either ammonium sulfate precipitation or by affinity isolation at a 1:400 dilution. In the indirect double sandwich ELISA, samples were probed with undiluted culture supernatant followed by alkaline phosphatase labeled goat anti-mouse antibody. Indirect nonsandwich ELISA was conducted in a similar fashion except the sample was diluted 1:100 in carbonate buffer and incubated at 4 C overnight before continuing the assay.

A dot-immunobinding assay with culture supernatant from hybridomas producing MAB to PMV was developed for detecting PMV in peanut foliar samples. Filter paper and nitrocellulose were examined as sample supports (8,18). Protein-A-peroxidase and goat anti-mouse alkaline phosphatase were compared for suitability as probes for detecting the antigen-antibody complex. Samples were prepared by grinding in Tris-buffered saline (TBS) and centrifuging at 10,000 *g* for 10 min. Samples were spotted on either filter paper or nitrocellulose and allowed to air dry. Samples on nitrocellulose were soaked in a 5% solution of Carnation nonfat dry milk for 30 min. Samples were then incubated in a 1:100 dilution of culture supernatant for 1 hr, rinsed three times with TBS, and incubated for 1 hr in either protein-A-peroxidase in TBS (2 μg/ml) or goat anti-mouse alkaline phosphatase (1:1,000 dilution in TBS). Samples were rinsed three times in TBS, then incubated in substrate buffer. The substrate for protein-A-peroxidase consisted of five parts TBS and 1 part of a 3 mg/ml-solution of 4-chloro-1-naphthol in methanol and 0.018 parts of 3% hydrogen peroxide (18). The substrate for alkaline phosphatase consisted of 5 mg of nitro blue tetrazolium in 15 ml of 0.1 M Tris buffer, pH 9.5, with 0.1 M NaCl and 5 mM $MgCl_2$, with 2.5 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 50 μl of N,N-dimethyl-formamide (12).

RESULTS AND DISCUSSION

MAB production and reaction with PMV isolates and other viruses. Fifty-four stable clones producing anti-PMV antibody were produced. All clones were determined to produce immunoglobulin of the IgG1 subclass. Three clones (10D4, 10A4, and 9A1) were selected to test for reaction to several PMV isolates and other viruses in an indirect double sandwich ELISA. Virus infected and control material was used at a 1:100 dilution. The three clones reacted strongly to the PMV-OK infected material, but not to uninfected pea or peanut tissue or material infected with wheat streak mosaic virus, peanut stripe virus, watermelon mosaic virus-1, potato virus Y, brome mosaic virus, wheat soilborne

mosaic virus, turnip mosaic virus, or tomato spotted wilt virus (Table 2).

A similar assay was conducted using various isolates of PMV. Virus infected and control material was used at a 1:100 dilution. Each experiment was repeated three times with each sample being assayed twice within the same plate. All clones reacted much more strongly to the virus infected material than to healthy pea tissue. Except for the reaction of 9A1 to PMV-IND and PMV-D, all clones gave ELISA values at least four times greater within 20 min to tissue infected with the various isolates than healthy tissue (Table 1). These data indicate that all the PMV isolates tested are serologically related because each clone reacted with all isolates. However, there were differences in the reaction of the different PMV isolates to a MAB. This may indicate that there are slight serological differences between the PMV isolates as there are slight differences among isolates in nucleic acid hybridization (2). All the isolates also reacted to polyclonal serum produced to the PMV-OK isolate. Other reports with polyclonal serum conclude that biologically different PMV isolates are serologically related (1,2).

To insure that the specificity of the assay was not due to the capture of the PMV antigens by the polyclonal serum, indirect nonsandwich ELISA was also conducted. In assays with viruses unrelated to PMV and with other PMV isolates, the results were the same as the indirect double sandwich ELISA. The MAB did not react with viruses unrelated to PMV but detected all PMV isolates.

Comparisons of ELISAs employing polyclonal antibodies and/or MAB. Six different formats of ELISA using either rabbit anti-PMV serum alone, MAB alone, or the two in combination were compared for detection of PMV in foliar samples. All assays readily distinguished between healthy and infected material (Table 3). The assay employing rabbit source antibody as both the coating antibody and probe antibody had the highest background reading compared with the other assays. MAB purified from ascitic fluid by either affinity isolation or ammonium sulfate precipitation and subsequently conjugated to alkaline phosphatase reacted similarly. Assays in which MAB were conjugated to alkaline phosphatase regardless of whether plates were coated with antibody from rabbit serum or MAB required three times longer to reach the same color intensity of the assays with the other ELISA formats.

The direct double sandwich ELISA in which plates were coated with MAB and samples probed with antibody from rabbit serum conjugated to alkaline phosphatase gave little background reaction and required the fewest steps. The indirect double sandwich ELISA in which plates were coated with antibody from rabbit serum and samples probed with unfractionated MAB culture supernatant fluid followed by alkaline phosphatase linked goat anti-mouse gave similar quick detection and did not require the isolation of MAB from the culture supernatant fluid. This format, however, requires an additional step during the assay.

These results are slightly different from those of Hill et al (9) with a radioimmunassay using both polyclonal rabbit antibody and mouse MAB to soybean mosaic virus. They found when the MAB was used as the coating antibody and polyclonal rabbit antibody used as the labeled antibody the assay was more sensitive than when the coating antibody was polyclonal rabbit antibody and the labeled antibody MAB. With the PMV system either approach gave satisfactory results. The conjugation of alkaline phosphatase to MAB purified by either affinity chromatography or ammonium sulfate precipitation reduced the speed of the reaction of the MAB in ELISA. This problem also has been encountered, to a greater degree, when MAB to potato leaf roll was conjugated to alkaline phosphatase (13).

Development of a dot-immunobinding assay with MAB. A dot-

TABLE 2. Reaction of peanut mottle virus-specific monoclonal antibodies (MAB) to selected viruses[a] in an indirect double sandwich enzyme-linked immunosorbent assay[b] (A_{405nm})

Sample	MAB from hybridoma clone		
	10D4	10A4	9A1
PMV-OK	1.284	0.505	1.456
WSMV	0.115	0.063	0.074
PStV	0.092	0.078	0.102
WMV-1	0.053	0.048	0.059
PVY	0.054	0.027	0.047
Healthy pea	0.042	0.049	0.050
BMV	0.074	0.074	0.071
WSBMV	0.053	0.028	0.048
TuMV	0.059	0.041	0.050
TmSWV	0.040	0.035	0.038
Phosphate-buffered saline	0.030	0.025	0.028
Healthy peanut	0.027	0.033	0.032

[a] PMV-OK = peanut mottle virus-Oklahoma isolate, WSMV = wheat streak mosaic virus, PStV = peanut stripe virus, WMV-1 = watermelon mosaic virus-1, PVY = potato virus Y, BMV = brome mosaic virus, WSBMV = wheat soilborne mosaic virus, TuMV = turnip mosaic virus.
[b] Plates were coated with rabbit polyclonal anti-PMV IgG at 1 μg/ml. Samples were added at a 1:100 dilution. Samples were probed with undiluted culture supernatant (MAB) followed by alkaline phosphatase labeled goat anti-mouse antibody. Values are averages of three experiments.

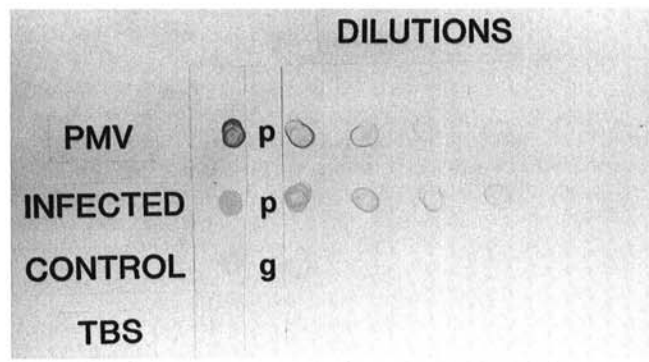

Fig. 1. Reaction of peanut mottle virus (PMV), PMV-infected peanut (infected), uninfected peanut (control), and Tris-buffered saline (TBS) in dot-immunobinding assay. Dilutions are twofold (left to right). In column 1: PMV spotted at 1,600 ng/ml; infected and control at 1 g per 4 ml of TBS. Color of spots are purple (p) in PMV and infected rows indicating positive reaction, and green (g) in control row indicating negative reaction.

TABLE 3. Reaction of rabbit anti-peanut mottle virus (PMV) serum (RAB) and anti-PMV monoclonal antibody (MAB) 10D4 in different combinations in ELISA[a] (A_{405nm})

Sample	MAB-E[b,c] MAB	RAB-E[d] MAB	MAB-E[c] RAB	MAB-E[e] RAB	RAB-E[d] RAB	Anti-MAB-E[f] RAB
PMV infected	1.312	1.222	1.122	1.197	1.117	1.133
Healthy control	0.026	0.022	0.015	0.013	0.111	0.016
Phosphate-buffered saline	0.023	0.012	0.016	0.016	0.019	0.007
Time for reaction (min)	60	20	60	60	20	20

[a] Plates were coated with rabbit polyclonal anti-PMV IgG or mouse ascitic fluid anti-PMV IgG at 1 μg/ml. Samples were added at a 1:100 dilution. Values are averages of three experiments.
[b] Upper line, probes; second line, coatings.
[c] Affinity purified MAB conjugated to alkaline phosphatase used at a 1:400 dilution.
[d] Ion-exchange purified rabbit anti-PMV IgG conjugated to alkaline phosphatase used at a 1:800 dilution.
[e] Ammonium sulfate precipitated MAB conjugated to alkaline phosphatase used at a 1:400 dilution.
[f] Alkaline phosphatase labeled goat anti-mouse antibody.

immunobinding assay using nitrocellulose as a sample support and goat anti-mouse alkaline phosphatase as a probe to detect the MAB-antigen complex gave the most satisfactory results (Fig. 1). Assays using filter paper as the sample support followed by either protein-A-peroxidase or anti-mouse alkaline phosphatase did not give as good a distinction between healthy and PMV-infected samples. This was also true when nitrocellulose was used as the sample support and the antigen-MAB complex was probed with protein-A-peroxidase. The dot-immunobinding assay was approximately 100–500 times less sensitive than the ELISA. With a double sandwich ELISA (MAB used to coat plate and alkaline phosphatase linked antibody from rabbit antiserum used as a probe) 1 to 2 ng/ml of PMV could be detected. In the dot-immunobinding assay the minimum detection level was between 100–500 ng/ml. The advantages of the dot-immunobinding assay are that it requires less time than the double sandwich ELISA, is less expensive to run when only a few samples need to be assayed, and requires only the MAB.

These results indicate the usefulness of using MAB to detect PMV in foliar tissue and confirm reports with polyclonal serum that biologically different isolates of PMV are serologically related. The MAB produced reacted to a broad range of PMV isolates and worked well in ELISA and a dot-immunobinding assay. The problem of the increased reaction time of ELISA when alkaline phosphatase conjugated MAB are used can be circumvented by using an assay in which samples are probed with alkaline phosphatase linked rabbit anti-PMV serum or by using a indirect double sandwich ELISA. This latter approach is advantageous because no special modification of hybridoma culture supernatant is required to run the assay.

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