SEROLOGICAL DETECTION OF WHEAT SOILBORNE MOSAIC VIRUS

BY

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SEROLOGICAL DETECTION OF WHEAT

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CHAPTER I

INTRODUCTION

Wheat soilborne mosaic virus (WSBMV) is among the earliest known viruses of wheat. Wheat soilborne mosaic disease was first recognized in 1919 in the central U.S.A. and the casual agent was characterized as a virus in 1923 (3,10,11,22). WSBMV was first reported in Oklahoma in 1952 (21) and is found in the north and north central parts of the state. The disease results in some yield reductions, but they are not as severe as in Plains States north of Oklahoma (7). WSBMV causes mosaic, stunting, and rosetting which are most prominent on early spring growth (1,22). The symptoms disappear with warming spring temperatures. Therefore, most yield reductions in Oklahoma occur during the years with a prolonged cool spring (7). WSBMV has caused up to a 50% loss of yield in wheat in the United States as well as in Japan and Italy (3,12,17).

The genome of WSBMV consists of two separately encapsidated RNA components. Component I has virions of 281 nm x 20 nm and Component II virions of 138 nm x 20 nm (1,18). Both components are needed for infection (17). WSBMV is naturally transmitted by Polymyxa graminis Led., a soilborne plasmodiophoraceous fungus, which often inhabits low-lying wet areas of the field (1,11,15,16,17,22). P. graminis is an obligate

root parasite of wheat which enters root hairs and epidermal cells of plant by its motile, biflagellate zoospores at high soil moisture (15,16,22). Langenberg and Giunchedi (8) showed that virus particles were not seen within fungal plasmodia, zoosporangia, or cystosori, but always were seen attached to the outer membrane of the vector. WSBMV may survive in soil for many years and is spread by wind, water, and any other factors that disperse infested soil (22). WSBMV can be transmitted artificially from infected to healthy plants by root washings that contain zoospores of P. graminis (7). Although WSBMV is not transmitted easily through sap, it can causes local lesions on mechanically inoculated Chenopodium species (1,16,22).

WSBMV was suggested to be serologically related to tobacco mosaic virus (TMV) and potato moptop virus (13,14). Hsu and Brakke, however, have shown with cDNA hybridization that WSBMV and TMV are not closely related (5). The lack of hybridization between WSBMV cDNA and TMV RNA indicates that WSBMV should be removed from the tobamovirus group and be put in the newly established group called furovirus (17).

Because the symptoms produced by WSBMV are very similar to symptoms produced by other viruses, a rapid method for determination of WSBMV infection would be beneficial. The purpose of the work presented in the following chapters was to develop approaches for the serological detection of WSBMV.

This thesis includes three manuscripts represented as independent chapters written in a format that will facilitate

their submission to a national scientific journal. Chapter II entitled "Comparison of a Filter Paper Dot-Immunobinding Assay and an Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Wheat Soilborne Mosaic Virus," shows the results of a comparison of these techniques and their use in the detection of WSBMV.

Chapter III, entitled "Production of Monoclonal Antibodies to Wheat Soilborne Mosaic Virus and Their Use in ELISA and Dot-Immunobinding Assays," describes the production of monoclonal antibodies and their use in ELISA and a dot-immunobinding assay for detection of WSBMV in infected plants. Chapter IV, entitled "Attempts at Detection of Wheat Soilborne Mosaic Virus by Agar Double-Diffusion," reports the unreliability of this test in detection of WSBMV.

The approval of writing the thesis in this manner as opposed to standard format is based on the Graduate College policy of accepting a thesis written in manuscript form and is subject to the Graduate College's approval of the major professor's request for a waiver of the standard format.

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CHAPTER II

Comparison of a Filter Paper Dot-Immunobinding Assay and an Enzyme-Linked Immunosorbent Assay (ELISA) for Detection of Wheat Soilborne Mosaic Virus

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ABSTRACT

A filter paper dot-immunobinding assay was compared to a double sandwich ELISA for the detection of wheat soilborne mosaic virus. Both assays were effective in detection of WSBMV in plant material. ELISA was a more quantitative assay than the filter paper dot-immunobinding assay. The filter paper dot-immunobinding assay was suitable for rapid detection of WSBMV and is especially suited to situations when only a few samples are to be run.

INTRODUCTION

The routine diagnosis of plant viruses often involves serological assays. Some of the simpler assays, e.g. agar double-diffusion, can not always be used with long rod shaped viruses or viruses that aggregate. Some of the more sophisticated assays, e.g. enzyme-linked immunosorbent assay (ELISA), require the special preparation of material. This

may not be suitable when a small number of samples are to be examined, or a small amount of antiserum is available.

Esen et al. (2) have described a simple and rapid dot-immunobinding assay for zein and other prolamins. This assay requires only filter paper discs as support media, unmodified antiserum, and commercially available protein-A-peroxidase and substrate. The binding of antibody to antigen is detected by protein-A-peroxidase which in the presence of 4-chloro-1-naphthol and hydrogen peroxide, forms a purple precipitate. Haber (3) has recently adapted this technique for detection of plant viruses.

Wheat soilborne mosaic virus, a rod shaped virus transmitted by Polymyxa graminis, is not readily detected in agar double-diffusion tests because the virus tends to aggregate. It was the purpose of research presented in this paper to compare the use of the filter paper dot-immunobinding assay and ELISA for the detection and quantification of WSBMV in infected material.

MATERIALS and METHODS

Virus Source

Certified seeds of wheat Triticum aestivum cv. Vona were sown in a field with a history of WSBMV disease. Plants were harvested after showing symptoms of WSBMV infection in early spring. The material was stored at -20C for later use.

Virus Purification

The method of Shirako and Brakke was used for purification of WSBMV (6). Approximately 180 g of frozen infected leaves were cut into small pieces and ground in 275 ml of 0.5 M grinding buffer (sodium borate 0.5 M, pH 9.0, with 0.001 M EDTA) in a blender. The extract was filtered through two layers of cheesecloth and centrifuged at 10,000 rpm for 10 min in a GSA rotor. The supernatant was strained through a Kimwipe and Triton X-100 was added to the filtrate to 2%. Each 24 ml of filtrate was cushioned by 6 ml of 20% sucrose in grinding buffer and centrifuged at 29,500 rpm for 2 hr at 4C in a Beckman type 30 rotor. Pellets were resuspended overnight in 0.05 M sodium borate buffer, pH 8.0, with 0.001 M EDTA, and were centrifuged for 10 min at 10,000 rpm at 4C in a SS-34 rotor. The supernatant was subjected to a second centrifugation with a cushion of 20% sucrose in 0.05 M sodium borate buffer, pH 8.0, with 0.001 M EDTA. The pellets were resuspended in 2 ml of distilled water and frozen at -20C overnight. After thawing they were centrifuged at 8500 rpm for 5 min in a Beckman microfuge. The supernatants were placed on 10-40% sucrose gradients in 0.05 M sodium borate buffer, pH 8.0, with 0.001 M EDTA and centrifuged in a Beckman SW 25.1 rotor for 2.75 hr at 24,500 rpm at 14C. The gradients were fractioned with an ISCO (Lincoln, NE 68504, USA) density gradient fractionator with an ultraviolet analyzer. The virus fraction was collected with the aid of an ISCO UA-5 absorbence monitor, mixed with buffer, and

centrifuged in a Beckman type 30 rotor for 2 hr at 29,500 rpm at 4C. The pellets were resuspended in 1 ml of the above buffer and stored at 4C until used. The yield of virus was 1.4 mg per 100 g of infected plant tissue.

Antiserum Production

A female New Zealand white rabbit was initially injected with 2 mg of WSBMV with an equal volume of Freund's complete adjuvant (1/2 subcutaneously, 1/2 intramuscularly). Then three weekly subcutaneous injections of 1 mg of WSBMV in Freund's incomplete adjuvant were given until a total of 5 mg of virus was administered. Ear bleedings were conducted weekly and antiserum titer was determined by microprecipitin test against purified WSBMV. When the antiserum titer fell below 512, subcutaneous boosters were given as above.

Enzyme-Linked Immunosorbent Assay (ELISA)

The procedure used for double sandwich ELISA was similar to that of Clark and Adams (1). The immunoglobulin G (IgG) fraction of WSBMV antiserum was isolated on DEAE-Trisacryl-M. ELISA plates were coated with 1.0 ug/ml of rabbit anti-WSBMV IgG in 0.05 M carbonate buffer, pH 9.6, with 0.003 M NaN_3 and incubated at room temperature for 2 hr. The plates were washed with phosphate buffered saline containing 0.05% Tween (PBS-Tween), and samples at a dilution of 1:100 in PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) were added. The plates were incubated at 4C overnight. Plates were washed

three times with PBS-Tween and then rabbit anti-WSBMV IgG that had been conjugated to alkaline phosphatase obtained from Sigma Chemical Co. (St. Louis, Mo 63178 USA) was added at a dilution of 1:200 in PBS-Tween containing 2% PVP and 0.2% ovalbumin. After an incubation period of 4-6 hr at room temperature, the plates were washed three times with PBS-Tween and the p-nitrophenyl phosphate in substrate buffer (4.8 ml diethanolamine and 0.1 g NaN_3 in 50 ml H_2O), pH 9.8, was added. The plates were then incubated 20 min before reading in a Bio-TEK EIA plate reader (Bio-TEK Instrument, Inc., Burlington, VT 05401, USA).

Filter-Paper Dot Immunobinding Assay

The steps of the filter-paper dot immunobinding assay were similar to those of Esen et al. (2). Plant material was ground in a 1:2 dilution in Tris-buffered saline, pH 7.4, (TBS: 0.05 M Tris-HCl and 0.20 M NaCl) and centrifuged for 10 min at 10,000xg. Two ul of supernatant was placed on 7 mm discs of Whatman No. 1 filter paper and allowed to air dry. Discs were then incubated in 200 ul of a 1:200 dilution of rabbit anti-WSBMV serum in TBS for 30-45 min on a shaker at 150 rpm at room temperature. Discs were rinsed three times in TBS and then incubated in 200 ul of a 2 ug/ml solution of protein-A-peroxidase for 15-30 min on a shaker at 150 rpm. Discs were then rinsed with TBS three times and incubated in 200 ul of substrate (5 ml TBS, 1 ml of a 3 mg/ml solution of 4-chloro-1-naphthol in methanol, and 18 ul of 3% hydrogen

peroxide) for about 10 min. Discs were rinsed in TBS to stop the reaction.

RESULTS and DISCUSSIONS

Optimization of Assays

In order to minimize non-specific reactions, the conditions for optimizing both assays were examined. With ELISA concentrations of 10, 1.0, and 0.1 ug/ml of IgG in coating buffer; dilutions of 1:10 and 1:100 of samples in PBS-Tween containing 2% PVP; and dilutions of 1:200, 1:800, and 1:3200 of alkaline phosphatase conjugated IgG were examined. A concentration of 1.0 ug/ml IgG in coating buffer, a dilution of 1:100 of samples, and a dilution of 1:200 of conjugated IgG gave the most satisfactory result. A 10 ng/ml suspension of purified virus could be detected and a linear response resulted as the virus concentration was increased.

With the filter paper dot-immunobinding assay, discs were initially spotted with 2 ul of various concentrations of purified WSBMV (8, 16, 32, 64, 128, 256, 512, 1024, 2048 ng/ml) and incubated in different dilutions of antiserum (1:50,100,200,400,800). As little as a 64 ng/ml suspension of virus was detected with a 1:50 to 1:200 dilution of antiserum (Fig. 1). When infected tissue was used, a 1:200 dilution of antiserum and a protein-A-peroxidase at 2 ug/ml gave the least amount of non-specific background reaction, so a 1:200 dilution of antiserum and protein-A-peroxidase at 2 ug/ml was used for all assays with plant material.

Discs spotted with material from either WSBMV infected wheat cv. Chisholm or control wheat were incubated in a 1:200 dilution of antiserum for 30, 45, or 60 min and in 2 ug/ml protein-A-peroxidase for 15, 30, or 45 min (Fig. 2). Satisfactory results were obtained with an incubation time of 30-45 min in antiserum and with an incubation time of 15-30 min in protein-A-peroxidase.

Detection of WSBMV

The filter paper dot-immunobinding assay was compared to ELISA for detection and quantification of WSBMV in different varieties of wheat. Twelve hard red winter wheat cultivars ranging in reactions to WSBMV were obtained from a field with a history of severe WSBMV (severe history) and a field with no previous disease although WSBMV was present (no disease history). Samples were collected and frozen at -20C until assays were run. Of the two assays, ELISA was more sensitive in discerning differences in virus concentration in resistant and susceptible varieties of wheat, but the filter paper dot-immunobinding assay could readily discern virus infected plants (Table 1).

CONCLUSIONS

Although both assays worked well for detection of WSBMV, situations exist when one assay could be more suitable than another. ELISA proved to be more suitable for relative quantification of virus in plant material. However, because

of the modification of antiserum required, e.g. fractionation of IgG and conjugation of IgG to alkaline phosphatase, this assay may not be useful when antiserum is in short supply, or only a few samples are to be run. The filter paper dot-immunobinding assay, however, is feasible for the rapid detection of WSBMV. This assay is very conservative of antiserum. The other advantages of the filter paper dot-immunobinding assay are rapidity (the assay can be completed in 2-3 hr), and economy (the assay is less expensive than ELISA). These are important considerations when only a qualitative diagnosis is necessary. However, when a large number of samples are to be examined and a quantitative assignment is required, ELISA may be the preferable assay.

TABLE 1: ELISA reading (A_{450}) and dot-immunobinding assay discs of wheat cultivars grown in a field with a history of severe wheat soilborne mosaic (severe history) and in a field with no previous history of the disease (no disease history).

	Severe History		No Disease History	
Variety[1]	ELISA	DIBA	ELISA	DIBA
Payne	1.43		0.21	
Newton	0.38		0.22	
TAM 105	1.77		0.23	
Sturdy	1.77		2.00	
Hawk	0.19		0.24	
TAM 101	1.70		0.57	
Chisholm	1.34		1.92	
Triumph 64	1.21		0.61	
Wings	1.40		1.80	
Sage	1.22		1.83	
Danne	0.91		1.88	
Vona	1.10		0.60	
PBS	0.06		0.06	
Control	0.08		0.08	

[1]Samples were collected 3-14-85 and frozen -20C until assayed.

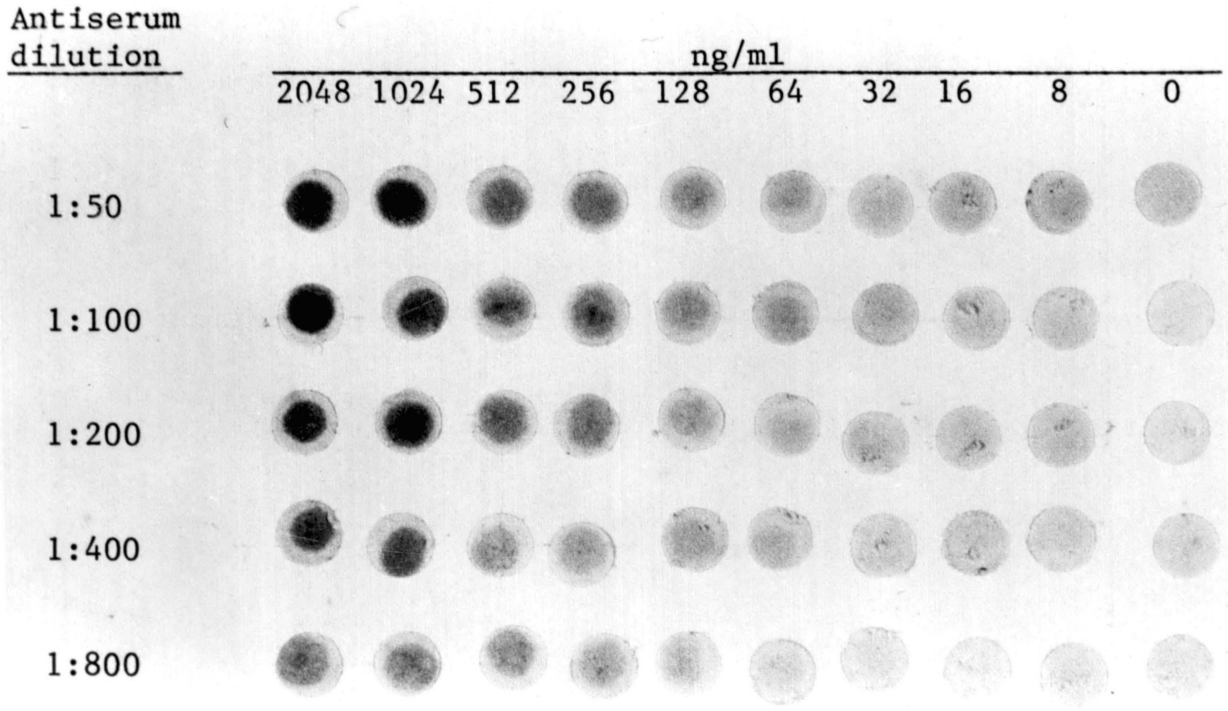

Figure 1. Detection of different concentrations of wheat soilborne mosaic virus (WSBMV), with different dilutions of antiserum to WSBMV in a filter paper dot-immunobinding assay.

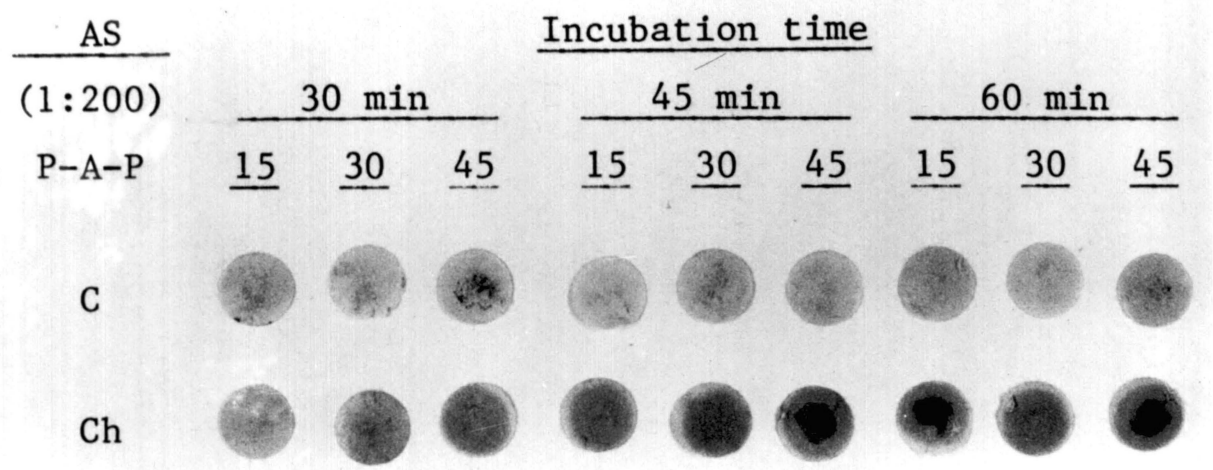

Figure 2. Optimization of time for incubation of paper discs in a 1:200 dilution of antiserum to wheat soilborne mosaic virus (WSBMV) and protein-A-peroxidase in a filter paper dot-immunobinding assay for detection of WSBMV (C = control, Ch = WSBMV infected wheat cv. Chisholm).

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CHAPTER III

Production of Monoclonal Antibodies (MCA) to Wheat Soilborne Mosaic Virus (WSBMV) and Their Use in Enzyme-Linked Immunosorbent Assay (ELISA) and Dot-Immunobinding Assays.

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ABSTRACT

Stable hybridoma cell lines secreting monoclonal antibodies (MCA) to WSBMV were produced by fusing spleen cells from BALB/c mice immunized with an isolate of WSBMV from Oklahoma to mouse myeloma cell line p3X63Ag8.653. Hybridoma clones produced antibodies of the IgG2a, IgG2b, and IgG3 subclasses. The MCA also reacted with an isolate of WSBMV provided by M. K. Brakke (University of NE), but not with wheat streak mosaic virus, tobacco mosaic virus, brome mosaic virus, tomato spotted wilt virus or a number of other potyviruses tested. The immunoreactivity of the MCA was compared to polyclonal rabbit serum for detection of WSBMV in ELISA and a dot-immunobinding assay. When ELISA was conducted with antibodies from a single source, assays with polyclonal antibodies or MCA had similar results. The MCA was superior to polyclonal serum in the dot-immunobinding assay. The IgG fraction of mouse ascities fluid containing MCA to

WSBMV was purified and conjugated to alkaline phosphatase, and used in different ELISA formats. The best results were obtained when a combination of MCA and polyclonal serum was used.

INTRODUCTION

Serology is a routine technique in identification, taxonomy, and diagnosis of plant viruses. However, serological analysis with conventional polyclonal antisera is often impaired by non-specific reactions of the antiserum (5). Antisera to the same antigen but from different animals can differ in quantity, quality, and reactivity with antigenic determinants (epitopes) of the antigen. Antisera from different bleedings of the same animal may also have these disadvantages (9,13). As a result, the use of sensitive serological techniques (e.g. enzyme-linked immunosorbent assay or ELISA) may be limited (13,16). When plant viruses are used as antigens, even after extensive purification, they are sometimes contaminated with host material. The use of these preparations as antigens may result in antiserum that contains undesirable antibodies against plant materials (16).

In 1975, Kohler and Milstein introduced a method of antibody production (i.e. production of monoclonal antibodies) that eliminated many of the problems associated with polyclonal antibodies (9,19,23). Monoclonal antibodies (MCA) have several advantages over polyclonal antisera. All antibodies produced from one hybridoma cell are identical and are

specific for only one antigenic determinant, facilitating the mapping of epitopes on protein molecules (2,5,9). This may aid in resolving problems associated with cross-reactivity of polyclonal antisera of two different viruses (2). MCA can be produced in a large quantity, so they can be used as standardized agents for serological diagnosis (5). The hybridoma cells can be preserved in liquid nitrogen for a long time, so antibody can be produced as needed (10). In addition, only a small amount of antigen is necessary to stimulate the immune response of mice (10).

The purpose of this research was to produce MCA to wheat soilborne mosaic virus (WSBMV) and to use them in detection of WSBMV by ELISA and dot-immunobinding assay.

MATERIALS and METHODS

Virus Source

WSBMV infected wheat plants Triticum aestivum cv. Vona were collected in spring 1984 from a field with a history of wheat soilborne mosaic at the Oklahoma State University Plant Pathology Farm at Stillwater, Oklahoma. Plants were stored at -20C until used.

Virus Purification

The method of Shirako and Brakke was used for purification of WSBMV (22). Approximately 180 g of frozen infected leaves were cut in small pieces and ground in 275 ml of 0.5 M grinding buffer (sodium borate 0.5 M, pH 9.0, with 0.001 M

EDTA) in a blender. The extract was filtered through a two-layered cheesecloth and centrifuged at 10,000 rpm for 10 min in a GSA rotor. The supernatant was strained through a Kimwipe and Triton X-100 was added to 2%. Each 24 ml of filtrate was cushioned by 6 ml of 20% sucrose in grinding buffer and centrifuged at 29,500 rpm for 2 hr at 4C in a Beckman type 30 rotor. Pellets were resuspended overnight in 0.05 M sodium borate buffer pH 8.0, with 0.001 M EDTA, and were centrifuged for 10 min at 10,000 rpm at 4C in a SS-34 rotor. The supernatant was subjected to a second centrifugation with a cushion of 20% sucrose in 0.05 M sodium borate buffer, pH 8.0, with 0.001 M EDTA. The pellets were resuspended in 2 ml of distilled water and frozen at -20C overnight. After thawing they were centrifuged at 8500 rpm for 5 min in a Beckman microfuge. The supernatants were placed on 10-40% sucrose gradients in 0.05 M sodium borate buffer, pH 8.0, with 0.001 M EDTA and spun in a Beckman SW 25.1 rotor for 2.75 hr at 24,500 rpm at 14C. The gradients were fractioned with an ISCO (Lincoln, NE 68504, USA) density gradient fractionator with an ultraviolet analyzer. The virus fraction was collected with the aid of an ISCO UA-5 absorbance monitor, then was mixed with buffer, and centrifuged in a Beckman type 30 rotor for 2 hr at 29,500 rpm at 4C. The pellets were resuspended in 1 ml of the above buffer and stored at 4C until used. The yield of virus was 1.4 mg per 100 g of infected plant tissue.

Myeloma Cell Line and Media

The mouse myeloma cell line p3X63Ag8.653 (653 cells), kindly provided by Dr. Ed Halk (Agrigenetics Corporation, Madison, WI 53716), was maintained at 37C with 5% CO_2 in RPMI 1640 medium (K.C. Biological, Inc., Lenexa, Kansas 66215 USA) supplemented with 10% horse serum (Hyclone Lab., Inc., Logan, Utah 84321 USA), 1.0 g/l $NaHCO_3$, 100 u/ml penicillin G, 0.10 g/l streptomycin sulfate, and 0.30 g/l L-glutamine (Sigma Chem. Co., St. Louis, MO 63178 USA). This myeloma cell line does not produce any endogenous Kappa or Lambda light chains or an immunoglobulin G (IgG) heavy chain (9,10). The suspensions of 653 cells were diluted with an equal volume of fresh medium on each of three days prior to fusion in order to insure log phase growth at the time of fusion.

After fusion, hybridomas were selected in HAT medium which consisted of 50 ul of aminopterin stock (3.5l/mg aminopterin per 10 ml of 0.1 N $NaHCO_3$), 1 ml of HT stock (0.1361 g hypoxanthine and 0.0388 g thymidine in 100 ml of water), and 100 ml of SP medium (40% RPMI 1640, 40% conditioned medium, 20% defined horse serum, and 0.05 mg/ml gentamicin) (18). Conditioned medium was prepared by adding 100 ml of a myeloma cell suspension to 500 ml of RPMI medium followed by incubation at 37C for 24 hr. The cells were pelleted at 400xg for 5 min and supernatants were used in making SP medium. The hybridoma cells were switched to HT medium (HAT without aminopterin) a week later. When cells were established they were grown in SP medium. For frozen storage, cells were

maintained in freezing medium (50% horse serum, 40% SP medium and 10% DMSO) and stored at -135C.

Immunization

Four BALB/c mice were injected intermuscularly with 750 ug of purified WSBMV. Two injections of 250 ug in Freund's complete adjuvant were made one week apart, followed by an injection of 250 ug of WSBMV in distilled water three days before cell fusion.

Cell Fusion

The procedure for cell fusion was adapted from that of Kohler and Milstein (19). Immunized mice were sacrificed and the spleens were removed aseptically. Spleens were "washed" by placing them sequentially in three petri dishes containing a balanced salt solution (BSS). BSS consisted of 8 g/l NaCl, 0.4 g/l KCl, 0.35 g/l $NaCO_3$, 1 g/l glucose, and 10 ml of a 0.2% phenol red solution. The spleens were removed from BSS and pressed gently through nylon mesh (0.1 mm^2) into 10 ml of serum-free RPMI 1640. Cells were centrifuged (400xg) for 7 min and the pellets were resuspended in 10 ml of serum-free RPMI 1640. An equal number of 653 cells were pelleted under 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 (3 ml/spleen) of 35% PEG (1000 daltons) in serum-free RPMI 1640, pH 7.8, and centrifuged at 190xg for 2 min. The cells were

left undisturbed in a 37C water bath for 6 min. The PEG was diluted out by two subsequent centrifugations in 20 and 30 ml of SP media, respectively, at 190xg for 8 min. The pellets were then resuspended gently in 20 ml of SP medium and divided into four T_{75} tissue culture flasks (Corning, NY 14831 USA). The volume in each flask was brought to 40 ml with SP medium, and flasks were incubated overnight at 37C. The contents of each flask were then centrifuged (400xg) for 5 min. The pellets were resuspended in approximately 125 ml of HAT medium, distributed into 26, 24-well tissue culture plates (Costar, Cambridge, MA. 02139 USA), and incubated at 37C (18).

Ten days after fusion, while cells were still in HT medium, clusters of cells with the typical morphology of hybridomas began to appear. At day 25, when the clones had expanded and the culture supernatants of most wells had turned yellow, the supernatants were collected, and the presence of antibodies against WSBMV was tested by indirect double sandwich ELISA.

Screening

Hybridomas were screened for antibody production by a modification of the ELISA method of Clark and Adams (3). Ninety-six well plates were coated with 1.0 ug/ml of rabbit anti-WSBMV immunoglobolin (IgG) in 0.05 M sodium carbonate buffer, pH 9.6, with 0.003 M NaN_3 and incubated at room temperature for 1-2 hr. The plates were washed three times

with PBS-Tween (phosphate buffered saline containing 0.05% Tween) and purified WSBMV at a concentration of 3 ug/ml in PBS was added to the wells and incubated overnight at 4C. In some cases hybridomas were screened using WSBMV infected plant material. Plant material was ground in either PBS-Tween containing 2% polyvinyl pyrrolidone (PVP) or PBS-Tween containing PVP with 1% SDS prior to adding to the plate and incubating overnight. Purified virus treated with SDS was also used in some screenings. Plates were then washed 3 times with PBS-Tween, blocked with 1% bovine serum albumin (BSA) for 10 min, and washed again. The culture supernatants were added to wells of ELISA plates and incubated for 2 hr at room temperature. After washing with PBS-Tween, alkaline phophatase conjugated goat anti-mouse IgG (Sigma), diluted to 1:1000 in PBS, was added to the wells and incubated for 2 hr at room temperature. After plates were washed, the substrate p-nitrophenyl phosphate disodium (Sigma) (1 mg/ml in 0.1 M glycine buffer, pH 10.4, containing 0.001 M $MgCl_2$ and 0.001 M $ZnCl_2$) was added to the wells and plates were incubated 20 min before the results were read. Pre-immune and immune sera obtained before and after immunization of mice with WSBMV, respectively, were used as negative and positive controls.

Cloning

Several positive hybridoma cell lines were cloned by using the soft agar cloning method. Approximately 50 ul of hybridoma cell suspension was placed in a 10 cm petri dish.

Ten ml of cloning medium (100 ml of SP medium, 2.4 ml Gibco amino acids with L-Glutamine, $5x10^{-2}$ M 2-mercaptoethanol, and 6.5 ml of 5% Difco agar in water) was added and swirled. Plates were incubated at 37C for 2 wk. Single cell colonies were picked and transferred into 1 ml of SP medium in 24-well tissue culture plates. The culture supernatants were retested for antibody production and positive wells were recloned a second and sometimes a third time. Antibodies produced by these clones were isotyped by ELISA.

Isotyping

A kit for identification of subclass specific antisera (Zymed Laboratories, Inc., So. San Francisco, CA 94080 USA) containing rabbit anti-mouse IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgA, IgM, Kappa light chain and lambda light chain was used for isotyping of MCAs by a triple sandwich ELISA. To each of nine wells of a WSBMV-coated plate 50 ul of culture supernatant was added and incubated 1 hr at 37C. After the plate was washed with PBS-Tween, 50 ul of the subclass-specific rabbit anti-mouse immunoglobins were added to each of the nine wells on the plate, and incubated for 1 hr at 37C. The plate was rinsed and the peroxidase-labelled affinity purified goat anti-rabbit IgG was added to all wells and incubated for 1 hr. The plate was rinsed and the substrate solution was added to all wells. The positive well corresponding to any of the subclass-specific immunoglobin was considered to have the same subclass of immunoglobin. After the spe-

cific subclass of MCA were identified, the clones were grown in T_{75} tissue culture flasks for mass production of antibodies. The culture supernatants were collected after centrifugation of clones in a clinical centrifuge, and stored at -20C.

Mouse Ascities Fluid Production

BALB/c mice were primed interperitoneally with 0.5 ml of 2, 6, 10, 14-tetramethyl pentadecane (Pristane) (Sigma) and a week later, about 10^7 hybridoma cells from four selected clones (8D67, 8A62, 5A24, 5BC4) were resuspend in 0.5 ml of BSS and injected into the peritoneal cavity of each mouse. The ascities fluids were usually collected with a 18-gauge needle 2-4 wk after injection of cells.

Immunoglobulin Purification

The IgG fraction of ascities fluid produced to clone 8D67 which produced IgG2a was purified with a Monoclonal Antibody Affinity Isolation Column (Hyclone). Five ml of ascities fluid was diluted to 100 ml with PBS and the affinity isolation agarose beads were added to this fluid. The mixture was gently stirred by magnetic stir bar for two hr, and then slowly poured into a scintered glass funnel into a 500 ml vacuum flask from which the fluid was drained. The beads were washed with approximately 500 ml of PBS until the effluent from the filter funnel was free of protein (less than 0.01 optical density at 280 nm). Then approximately 200

ml of eluting buffer (0.3 M acetic acid containing 0.85% sodium chloride) was slowly added to the beads. The elution of antibodies from the affinity beads was done with a vacuum with suction of 3 inches of Hg. To decrease the time of exposure of antibodies to an acid condition, 2.4 g of solid Trisma base (Sigma) was placed into the eluting flask prior to the elution step. The contents of flask were immediately dialyzed first against 0.2 M saline pH 8.5 and then against PBS, each three times. The IgG solution was collected and concentrated by an Amicon ultrafiltration cell (molecular cut off of 80,000 daltons) with 50 pounds pressure. The IgG solution was collected and the optical density was determined by spectrophotometry. The IgG was retested by indirect double sandwich ELISA for reactivity and antibody titer.

Enzyme-Labeled Purified IgG

The affinity isolated IgG was labeled with alkaline phosphatase (Sigma, No. P-2276) by the method described by Clark and Adams (3). Two thousand units of enzyme were resuspended in 0.8 ml of purified IgG (1.4 mg/ml). The solution was dialyzed three times against PBS. The dialyzed solution was mixed with glutaraldehyde to a final concentration of 0.06%, and incubated at room temperature for 4 hr. The solution was then dialyzed three times against PBS and stored at 4C. The reactivity of conjugated IgG was tested by double sandwich ELISA.

Infecting Wheat by Root Washing

Clumps of the wheat cv. Vona with symptoms of WSBMV were removed in March 1985 from a field with a history of wheat soilborne mosaic, for use as source of inoculum for root washing experiments. Plants were grown in soil in 2 ice chests at 4C, 80 cm under a bank of eight Gro-Lux lights set for a 12 hr photoperiod. Plants were watered weekly with 500 ml of tap water.

Certified seeds of cv. TAM 105 were planted in 4 rows in autoclaved metal flats containing a mixture of sandy loam soil and peat. The flats were kept in a growth chamber at 15C with a 12 hr photoperiod. They were inoculated approximately six days after planting when coleoptiles of most plants had emerged.

The preparation of inoculum from source plants was adapted from the method of Bockus and Niblett (1). The source plants were removed from soil, washed completely, and root and crown tissues collected. Two hundred sixty-seven grams of root and crown tissue were placed in a 2 L beaker containing 1335 ml of distilled water, and the temperature of this mixture was adjusted to that of the test plants by placing the beaker in the growth chamber. Each flat was flooded with 667 ml of root washing 1,3,5 and 8 hr after the time root and crown tissues were soaked in water. Thus each flat recieved a total of 2668 ml of root washing. During and after inoculation, the flats were kept in a growth chamber. Thereafter, the foliage of plants was collected once a week

for 8 wk and stored at -20C and then tested by double sandwich ELISA for presence of the virus (17).

The flats of infected wheat were used as a virus source to inoculate five hard red winter wheat cultivars, using the root washing method described above. There were two replicates of flats, and each flat had two rows of each cultivar, arranged randomly. Approximatly 5 g of foliar samples were taken every week after inoculation for a period of 8 wk, stored at -20C, and used for comparison of MCA and polyclonal antibodies by ELISA.

Dot-Immunobinding Assay with Monoclonal Antibodies

The method used for this experiment was a modification of the procedure of Esen, et al. (6,8). Fresh plant material obtained from growth chamber (WSBMV infected and non-infected wheat cv. TAM 105) was ground (1:2) in Tris-buffered saline, pH 7.4, (TBS, 50 mM Tris-HCl and 200 mM NaCl) and centrifuged for 10 min at 10,000xg. Four microliters of the supernatants, and purified virus as a control, were placed on a 5x5 cm sheet of nitrocellulose paper and allowed to air dry. The unbound sites were blocked for 20 min at room temperature in a solution of 5% Carnation non-fat dry milk (Los Angeles, CA 90036, USA) in TBS and incubated on a shaker at 150 rpm. After three rinses in TBS, the nitrocellulose sheet was placed in hybridoma culture supernatant diluted 1:100 in TBS. After agitation for 1 hr at room temperature, the nitrocellulose sheet was rinsed three times with TBS and then placed

in a 1:100 dilution of alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) in TBS for 1 hr. The nitrocellulose sheet was then rinsed with TBS and placed in substrate buffer for about 5 min and then washed. Substrate solution was made by heating 15 ml of AP buffer (0.1 M Tris-HCl, pH 9.5, with 0.1 M NaCl and 5 mM $MgCl_2$), (20) to 35 C, adding 5 mg Nitro Blue Tetrazolium (NBT) (Sigma), and vortexing vigorously for 3 min. Then, 2.5 mg of 5-bromo-4-chloro-3 indolyl phosphate (BCIP) (Sigma) in 50 ml of N, N-dimethylformamide was added dropwise to the NBT solution.

The dot-immunobinding assay was compared to a similar assay with rabbit polyclonal serum. The procedure used was the same except rabbit serum diluted 1:200 was used instead of culture supernatant, and alkaline phosphatase conjugated goat anti-rabbit IgG was used instead of conjugated anti-mouse IgG.

RESULTS and DISCUSSIONS

Hybridoma

From a total of 610 wells that were initially plated, 120 were found to be positive to WSBMV by indirect double sandwich ELISA. Cells from positive wells were expanded in SP medium.

Cloning

Cells from positive wells were cloned in soft agar and reactivity of antibodies was retested after each cloning by

ELISA with purified virus and WSBMV infected plant tissue (Table 1). Most clones reacted with purified virus and infected plant material regardless of the treatment with SDS. Additional clones reacted in ELISA when purified virus and infected material was treated with SDS. Other clones reacted only with infected material. Additional clones reacted with only infected material if it was treated with SDS.

Immunoglobulin Subclass Identification

The clones that reacted strongly with both purified virus and infected plant tissue were chosen and the IgG subclass was determined using individual isotype specific antiserum by triple sandwich ELISA (Table 2). All clones possessed a kappa light chain. Isotyping by agar-double diffusion was attempted, but was not successful.

Antibody Production

Four cell lines (8D67, 8A62, 5A24, and 5BC4) were chosen for expansion and production of antibodies in flasks and in mice. Clone 5A24 stopped production of antibodies after eleven months of maintenance in culture, while the other clones continued to produce antibodies. Antibodies from clones 8D67, 8A62, 5A24, and 5BC4 were precipitated three times by ammonium sulfate. Culture supernatant from each clone had a titer of 10^5 in indirect double sandwich ELISA.

Mouse Ascities Production

Cell lines 8D67, 8A62, 5A24, and 5BC4 were grown in flasks and then injected into mice. Clone 8D67 produced 10 ml of ascities fluid. Hybridomas 8A62 and 5A24 produced solid tumors, so only 3-4 ml ascities fluid was produced per mouse. Clone 5BC4 never produced any ascities fluid. The titer of ascities fluids was 10^6 as determined by indirect ELISA. The subclass of antibodies of ascities fluid were retested; they were the same as those of culture fluid.

Reaction of Monoclonal Antibodies to WSBMV to Other Plant Viruses

MCA to WSBMV were tested for reaction to other plant viruses by an indirect double sandwich ELISA. Plant tissues infected with wheat streak mosaic virus (WSMV), brome mosaic virus (BMV), two isolates of peanut mottle virus (PMV, PMV-M), tomato spotted wilt virus (TmSWV), watermelon mosaic virus-1 (WMV-1), peanut stripe virus (PStV), turnip mosaic virus (TuMV), tobacco mosaic virus (TMV), and potato virus Y (PVY) and an isolate of WSBMV from Nebraska provided by Dr. Myron Brakke were tested. Plant materials were diluted 1:100 and samples were tested by indirect ELISA. The MCAs to WSBMV reacted with the Nebraska WSBMV isolate, but not with any other viruses (Table 3).

Comparison of Monoclonal Antibodies and Polyclonal Antibodies for Detection of WSBMV by ELISA

Samples collected weekly from infected plants grown in the growth chamber were tested by direct double sandwich ELISA. Samples collected 4 wk after inoculation showed no infection with WSBMV, but samples collected from week 5 to 8 showed an increase in concentration of virus. The plants from these flats were used as sources for inoculating the wheat varieties. Samples of all five varieties collected at week 6 were tested by ELISA to determine the variety with the highest concentration of WSBMV. The cv. Sage was chosen for later experiments. Samples of Sage collected at all weeks were tested with clones 8D67, 8A62, 5A24, and 5BC4. Clone 8D67 had the strongest reaction. Healthy and infected plant material harvested at weeks 1, 3, 5, and 7 was chosen to compare clone 8D67 and polyclonal rabbit antiserum in ELISA for detection of WSBMV (Table 4). Both antibody sources reacted with similar intensity. The polyclonal serum reacted more quickly, but the MCA had lower background.

Comparison of Monoclonal Antibodies and Polyclonal Antibodies by Dot-Immunobinding Assay

Attempts to perform the dot-immunobinding assay with MCA with filter paper as a support medium failed. Using either protein-A-peroxidase, peroxidase conjugated goat anti-mouse IgG, or alkaline phosphatase conjugated goat anti-mouse with MCA resulted in very strong background. When nitrocellulose paper was used instead of filter paper, strong

background was observed when protein-A-peroxidase or peroxidase conjugated goat anti-mouse IgG were used. However, when alkaline phosphatase conjugated anti-mouse IgG was used, a sharp distinction between infected and healthy plant material was evident (Fig. 1). When polyclonal antiserum was used and alkaline phosphatase conjugated anti-rabbit IgG for detection of WSBMV used to detect the antigen-antibody complex, a distinction between infected and healthy plant material was not evident (Fig. 2).

Enzyme-Labeled Monoclonal Antibodies

The IgG purified by Monoclonal Affinity Isolation Column had a titer of 10^6 in indirect ELISA and a concentration of 1.17 mg/ml. The purified IgG was labeled with alkaline phosphatase and the reactivity was tested by double sandwich ELISA. Plates were coated with 0.1, 1.0, and 10 ug/ml of MCA IgG and labeled antibody was added at dilutions of 1:200, 1:800, and 1:3200. Satisfactory results were obtained using 0.1 ug/ml of purified IgG for coating, a 1:200 dilution of enzyme conjugated IgG, and a 1:10 dilution of infected plants.

Use of Combinations of Monoclonal Purified IgG and Rabbit Purified IgG in ELISA

To develop an assay incorporating the advantages of MCA and polyclonal antibodies, ELISA plates were coated with 1.0 ug/ml of rabbit IgG and probed with a 1:200 dilution of enzyme-conjugated rabbit IgG , coated with 0.1 ug/ml of MCA

purified IgG and probed with enzyme conjugated rabbit IgG in a dilution of 1:200, coated with 1.0 ug/ml of rabbit purified IgG and probed with enzyme-conjugated MCA purified IgG in a dilution of 1:200, or coated with 0.1 ug/ml of MCA purified IgG and probed with a dilution of 1:200 enzyme-conjugated MCA purified IgG (Table 5). The best result was obtained when a combination of two antibody sources was used, i.e. plate coated with IgG fraction of rabbit serum and probed with enzyme labled IgG fraction of MCA or vice versa. When MCA were used alone the reaction was very slow. With polyclonal antibodies alone, a strong background appeared as the assay developed.

CONCLUSIONS

Several hybridoma cell lines secreting monoclonal antibodies to WSBMV were produced. In an indirect double sandwich ELISA, the only additional virus which reacted with MCA to WSBMV was an isolate of WSBMV from Nebraska. Other viruses, e.g. WSMV, TMV, BMV, TmSWV, and a number of other potyviruses did not react significantly with the clone. This shows that WSBMV dose not share this antigenic determinant with the other viruses tested. The clones that reacted only with SDS treated material may only react with internal epitopes of the virus (10).

The reactivity of MCA was compared to polyclonal rabbit serum for detection of WSBMV in ELISA and a dot-immunobinding assay. In ELISA, using IgG fraction of rabbit serum for

coating the plates, two antibody sources reacted with similar intensity. A combination of MCA and polyclonal serum proved superior for detection of WSBMV than assays employing only one type of antibody. With dot-immunobinding assay, MCA reacted very well with WSBMV infected plant with a good distinction between infected and healthy plant material and without any background.

Table 1: Reaction of second clones with purified WSBMV and WSBMV infected material treated or not treated with SDS.

Positive reaction with purified virus and infected material		Positive reaction with infected material only	
with SDS	without SDS	with SDS	without SDS
8D61	8D61	2D61	----
8D65	8D65	3D6	----
5A1	5A1	5A22	5A22
2A2	2A2	5A23	----
8A62	----	2B6	2B6
8D62	----	8B12	----
8D66	----		
8D5	----		
8D63	8D63		
8D67	8D67		
2D62	2D62		
8D64	8D64		
10A4	10A4		
5A24	5A24		
8D68	8D68		
2D63	2D63		
5BC4	5BC4		

Table 2: Antibody isotypes of monoclonal antibodies from hybridomas to wheat soilborne mosaic virus.

Cell line	Antibody isotype
8D61	IgG_{2a}
8D62	IgG_{2a}
2D62	IgG_{2a}
8D67	IgG_{2a}
8D64	IgG_{2a}
10A4	IgG_{2a}
8D65	IgG_{2a}
2A4	IgG_{2a}
2D63	IgG_{2b}
8D63	IgG_{2b}
5A1	IgG_{2b}
5A24	IgG_{2b}
8D68	IgG_{2b}
5BC4	IgG_3
8A62	IgG_3

Table 3: Reaction of clone 8D67 to selected plant viruses in indirect double sandwich ELISA (A_{405}). Each values average of five readings.

Virus	A_{405}
Buffer control (PBS)	0.034
Healthy control	0.029
WSMV	0.060
BMV	0.084
PMV	0.052
TmSWV	0.031
WMV-1	0.041
PStV	0.039
TuMV	0.077
TMV	0.033
PVY	0.033
WSBMV (Nebraska isolate)	0.421
WSBMV (Purified virus, Oklahoma isolate)	0.269

Table 4: Comparison of a double sandwich ELISA with polyclonal rabbit serum and an indirect double sandwich ELISA (A_{405}) with a combination of polyclonal rabbit serum and mouse monoclonal antibody for detection of WSBMV. Each value is average of five readings.

Cultivars	Weeks post inoculation															
	1				3				5				7			
	PCA[1]		MCA[2]		PCA*		MCA**		PCA		MCA		PCA		MCA	
	I[3]	H[4]	I	H	I	H	I	H	I	H	I	I	I	H	I	H
Sage	0.08	0.04	0.004	0.003	0.120	0.04	0.050	0.003	0.030	0.040	0.020	0.003	0.230	0.040	0.190	0.003
TAM 105	-0.04	0.01	0.030	0.005	0.030	0.01	0.007	0.005	0.240	0.010	0.200	0.005	0.160	0.010	0.160	0.005
Danne	0.06	0.02	0.020	0.009	0.10	0.02	-.001	0.009	0.152	0.020	0.200	0.009	0.340	0.020	0.226	0.009
Newton	0.05	0.03	0.010	0.010	0.05	0.03	0.02	0.010	0.200	0.030	0.210	0.01	0.020	0.030	0.010	0.010
Hwk	0.03	0.06	0.020	0.005	0.09	0.06	-.005	0.005	0.030	0.060	0.020	0.005	0.120	0.060	-.005	0.005

1. IgG fraction of polyclonal serum probed with rabbit serum bound to alkaline phosphatase.

2. IgG fraction of polyclonal serum probed with monoclonal antibodies followed by goat anti-mouse bound to alkaline phosphatase

3. Infected plant material

4. Healthy plant material

* Time for all reactions was 35 minutes

** Time for all reactions was 2 hours

Table 5: Reaction of wheat soilborne mosaic virus (WSBMV) polyclonal and monoclonal antibodies in different ELISA (A_{405}) formats. Each value is average of five readings.

Coating antibodies / Probe antibodies	MCA[1] / MCA-E[3]			MCA / RAB-E[4]			RAB[2] / MCA-E			RAB / RAB-E			RAB / MCA[5]
Time for reaction (min)	30	45	105	30	45	105	30	45	105	30	45	105	45
WSBMV Infected Plant	0.11	0.23	0.80	0.70	1.23	2.00	0.21	0.41	1.11	1.60	2.00	2.00	0.80
Healthy Control	0.07	0.00	0.04	0.06	.008	0.00	.004	.002	.234	0.91	1.40	2.00	0.03
PBS	0.04	0.00	0.01	0.03	0.00	.005	0.02	.006	.232	0.90	1.32	2.00	0.05

1. IgG fraction of ascites fluid

2. IgG fraction of polyclonal serum

3. IgG fraction of ascities fluid bound to alkaline phosphatase

4. IgG fraction of polyclonal serum bound to alkaline phosphatase

5. IgG fraction of polyclonal serum probed by IgG fraction of ascities fluid and followed by goat anti-mouse bound to alkaline phosphatase

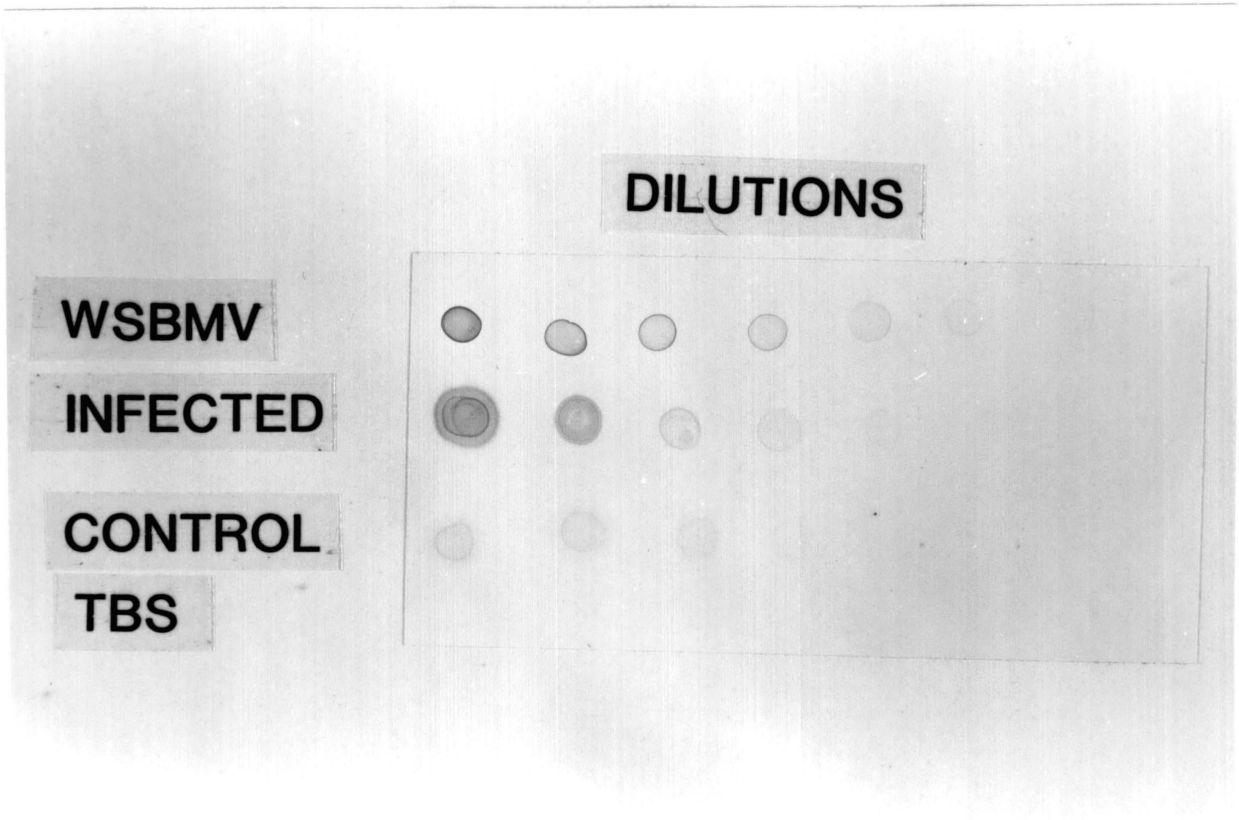

Figure 1. Detection of wheat soilborne mosaic virus (WSBMV) by dot-immunobinding assay with monoclonal antibodies in pure preparation of WSBMV and infected plants (dilutions: 1000,500,200,100,50, 10,5,1 ng/ml of purified virus and 1:2,4,8,16, 32,64,128,256 of infected and healthy plant materials).

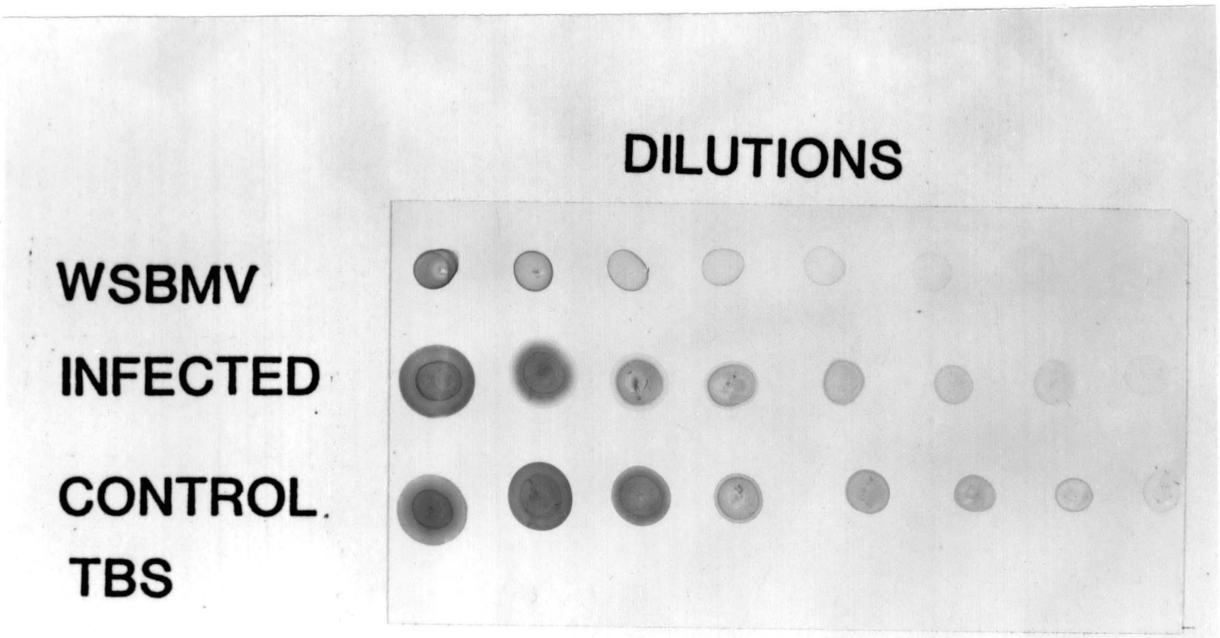

Figure 2. Detection of wheat soilborne mosaic virus (WSBMV) by dot-immunobinding assay with polyclonal antiserum in pure preparation of WSBMV and infected plants (dilutions: 1000,500,200,100,50, 10,5,1 ng/ml of purified virus and 1:2,4,8.16, 32,64,128,256 of infected and healthy plant materials).

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CHAPTER IV

Attempts at Detection of Wheat Soilborne Mosaic Virus by Agar Double-Diffusion.

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ABSTRACT

Agar double-diffusion tests were performed with healthy and infected plant material to detect wheat soilborne mosaic virus (WSBMV). The antiserum to WSBMV reacted to healthy as well as infected plant sap. Antiserum was cross-reacted with host protein, but when plant sap was treated with SDS and incubated with the antiserum, the false-positive reaction was still observed. SDS treated healthy plant material also reacted with BMV, TMV, and WSMV antisera. From these results, it is concluded that agar double-diffusion is not a suitable test for detection of WSBMV.

INTRODUCTION

Immunodiffusion tests have been used extensively in plant virology. Generally, these tests have been extremely useful tools in virus diagnosis and determination of virus relationships (6). They can be used with crude sap, clarified sap, and purified virus preparations (2). Immunodiffu-

sion tests involve the use of agar or agarose in which the antigens and antibodies diffuse. Where the antigens and antibodies meet at optimal concentrations, a line of precipitation is formed. The most useful immunodiffusion test is the agar double-diffusion test devised by Ouchterlony in 1948 (2,4). This test has many advantages which include sensitivity, and ability to separate mixtures of antigens and antibodies according to the size, concentration, and diffusion coefficient (1,2).

The agar double-diffusion test has some disadvantages, too. One major limitation of this test is that filamentous viruses or those which have a tendency to aggregate can not readily diffuse through agar gels. Degradation of virus aggregates or virions by dissociating agents such as sodium dodecyl sulfate (SDS) has been shown to give satisfactory results (1,6). However, degradation by dissociating agents may change the antigenic character of viruses by exposing epitopes that are normally internal (2).

The purpose of this research was to determine if agar double-diffusion tests could be used in detection of wheat soilborne mosaic virus (WSBMV).

MATERIALS and METHODS

Virus Source

Infected wheat plants Triticum aestivum, cv. Vona were obtained in early spring from a field with a history of WSBMV disease, and were stored in -20C.

Virus Purification and Antiserum Production

The method of Shirako and Brakke (8), was used for purification of WSBMV. Purified virus was stored at 4C.

For antiserum production a female New Zealand white rabbit was initially injected with 2 mg of the WSBMV with an equal volume of Freund's complete adjuvant, 1/2 subcutaneously, and 1/2 intermuscularly. Then three weekly subcutaneous injections of 1 mg/ml of WSBMV in Freund's incomplete adjuvant were given until a total of 5 mg of virus was administered. Ear bleedings were conducted weekly and antiserum titers were determined by a microprecipitin test against purified WSBMV. When the antiserum titer fell down 512, subcutaneous boosters were given as above.

Agar Double-Diffusion

The procedure used was similar to one devised by Ouchterlony (4). Eight tenths gram of Ionagar No. 2 and 0.25 g of NaN_3 were added to 100 ml of PBS (0.01 M potassium phosphate, pH 7.4, 0.15 M NaCl) and after heating to boiling in a microwave oven, the gel was poured in 10 cm plastic petri dishes in 2 mm layers and allowed to solidify. Six wells in the shape of a regular hexagon of 6 cm circumference and a central well were cut and gel plugs were removed by suction. To the outside wells different antigens or different antigen dilutions were added, and to the center well, the desired antiserum was added. The plates were stored in a humidified

chamber at room temperature to prevent desiccation. Within 48 hr to 1 wk the plates were examined for precipitin lines.

RESULTS and DISCUSSIONS

WSBMV infected wheat material cv. Sage was ground in PBS (0.5 g/ml), and serial twofold dilutions of the sap were made to 1:32 with PBS. WSBMV antiserum was diluted to 1:2 and 1:4 in PBS to optimize the antiserum dilution. Dilutions of plant sap or antiserum were placed in the appropriate wells. After 48 hr incubation, positive reactions were seen with the 1:2 dilution of antiserum with different dilutions of infected plant material (Fig. 1-a). However the same result was obtained with healthy plant material (Fig. 1-b). To remove antibodies against plant proteins which may have been co-purified with WSBMV, the antiserum was cross-reacted with healthy plant sap. A 0.4 ml of the 1:2 dilution of healthy plant sap was placed in microfuge tube and serial twofold dilutions of healthy plant sap were made in PBS. An equal volume of WSBMV antiserum (0.2 ml) was then added to each tube, mixed well and then incubated overnight at 4C. These tubes were then centrifuged (3000xg) for 10 min. The supernatants of these tubes were used as cross-reacted antiserum, and allowed to react with different dilutions of healthy plant sap in PBS in agar double-diffusion plates (7). There were no reactions between the cross-reacted antiserum dilutions with any of healthy plant sap dilutions (Fig. 1-c). The same experiment was repeated with infected plant material

(three different cultivars including Sage) but the same result was obtained (Fig. 1-d).

To see if purified WSBMV would react with WSBMV antiserum in agar double-diffusion, different concentrations (0.2, 0.1, 0.05, 0.025, 0.0125 mg/ml) of the virus were made in PBS and tested with WSBMV antiserum in an agar plate. There were no reactions (Fig. 1-e). This could be due to the size of the virus or to aggregation which makes it difficult for the virus diffuse through agar gels (2,4,5). To eliminate this possibility, agar was prepared in dissociation buffer (0.02 M Tris-HCl, 0.1 M NaCl, 0.01 M EDTA, 1.0% sodium dedecyl sulfate (SDS), and 100 ug/ml of bentonite, pH 9.0). SDS breaks the particles into smaller fragments and makes them diffusible into agar gels (2,5). Different dilutions of purified WSBMV were made in dissociation buffer and were tested with cross-reacted antiserum. Reactions were observed between the cross-reacted antiserum and virus treated with dissociation buffer (Fig. 1-f). To see the reaction of infected and healthy plant material in dissociation buffer, a serial dilution of each was made in dissociation buffer and these were tested with cross-reacted antisera in two different agar plates. A positive reaction occured with infected plant material (Fig. 1-g) as well as healthy material (Fig. 1-h). To see if this phenomenon is associated with only WSBMV antiserum, the healthy plant sap was reacted with brome mosaic virus (BMV), tobacco mosaic virus (TMV), and wheat streak mosaic virus (WSMV) antisera in an agar plate.

None of these antisera reacted with healthy plant sap dilutions. The healthy plant material was then prepared with dissociation buffer and tested with BMV, TMV, and WSMV antisera in an agar made with dissociation buffer. The plant material reacted with all of the antisera. The reason for this phenomenon is unclear. Similar results were obtained by Mink, et al. when they found that some apple tip leaf antigens reacted with tomato ring spot virus antisera in ELISA (3).

CONCLUSION

Agar double-diffusion is a useful test for identification of most plant viruses from crude sap, clarified sap, and purified preparations (2). However, this assay was not a suitable test for detection of WSBMV. Agar double diffusion works well with purified virus treated with SDS. However, fragmentation resulting from treating with SDS may change the antigenic character of the virus, exposing epitopes that are normally internal (2). In addition, using purified virus does not serve the purpose of developing a quick detection of WSBMV in diseased plants, especially when the sample is limited in amount.

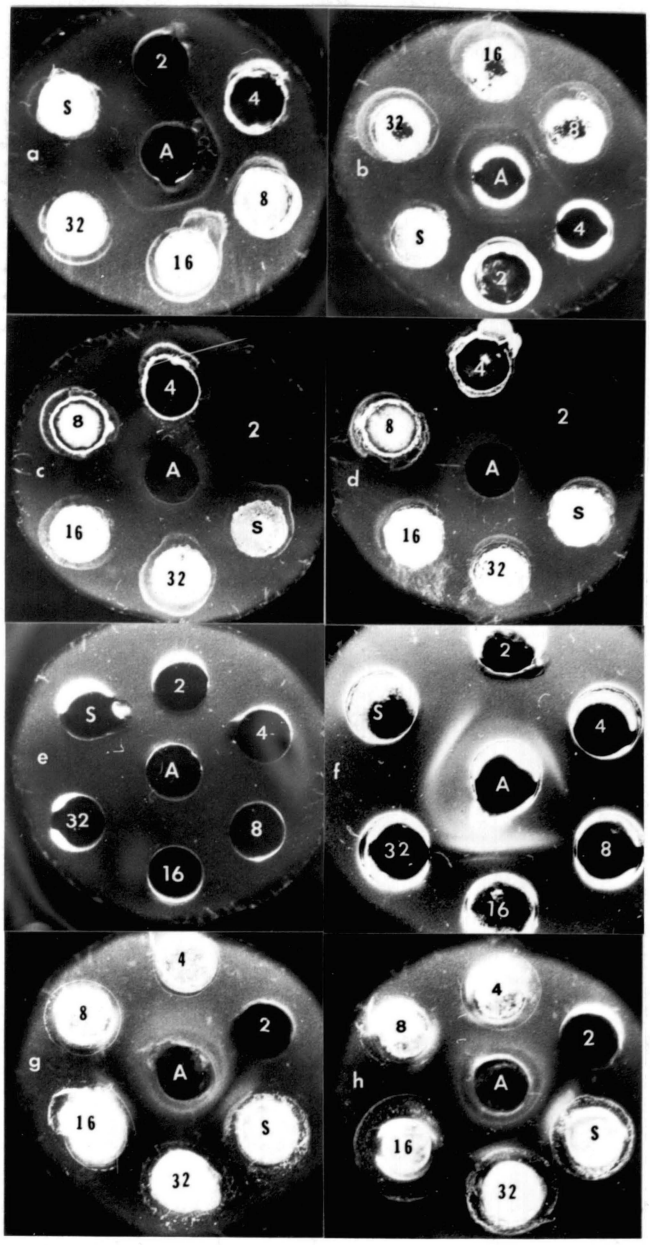

Figure 1. Agar double-diffussion test in agar plates with and without SDS (see text for further details). A = antiserum; S = PBS; 2,4,8,16,32 = 1:2,4,8, 16,32 dilutions of plant materials.

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