

Use of Monoclonal Antibodies in Detection of Tomato Spotted Wilt Virus

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Accepted for publication 28 June 1988 (submitted for electronic processing).

ABSTRACT

Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. *Phytopathology* 79:61-64.

A stable hybridoma cell line secreting monoclonal antibody (MAB) to tomato spotted wilt virus (TSWV) was produced by fusing spleen cells of immunized mice and mouse myeloma cell line P3X63Ag8.653. MAB from hybridoma clone 1-7B4 produced antibody of the IgG2b subclass. MAB 1-7B4 reacted to five isolates of TSWV, but did not react to 10 other viruses tested. The immunoreactivity of the MAB was examined in a protein-A

sandwich enzyme-linked immunosorbent assay (ELISA), a double-antibody sandwich ELISA, and a dot-immunobinding assay. Isolated nucleocapsid protein reacted with the MAB in ELISA, but the envelope-associated proteins did not. To our knowledge, this is the first report of the production and utilization of a MAB to detect an enveloped plant virus.

Tomato spotted wilt virus (TSWV) is the only member of the tomato spotted wilt virus group, but there is a large number of strains of the virus (4,9). The virus has a wide host range and is transmitted by thrips. TSWV is enveloped with an isometric morphology and a diameter of about 85 nm. Three virus-specific glycoproteins of 52 kDa, 58 kDa, and 78 kDa are present in the virus envelope, and the nucleocapsid has a protein of 29 kDa (14). Because the virus is enveloped, purification of the virus and routine production of highly specific polyclonal antiserum is difficult (5). Monoclonal antibody (MAB) technology provides a means to produce a potentially unlimited supply of highly specific uniform antibody (10) that is useful in the detection of plant viruses (6,7). To our knowledge, however, this technology has not been used to produce antibodies for the detection of enveloped plant viruses. The purpose of this study was to produce and characterize MAB to the enveloped plant virus TSWV, test the reaction of selected MAB to isolates of TSWV, and test the utility of TSWV-specific MAB in diagnostic assays.

MATERIALS AND METHODS

Viruses and purification. The TSWV isolate (TSWV-OK) used for MAB production was maintained in *Datura stramonium* L. and purified according to the method of Mohamed et al (14). Other isolates of TSWV were obtained from H. Scott, University of Arkansas (TSWV-AR); B. Reddick, University of Tennessee (TSWV-TN); and J. Cho, University of Hawaii (TSWV-T1 and TSWV-T2). These isolates were also maintained in *D. stramonium*. In addition, the TSWV-T1 and TSWV-T2 isolates were maintained in *Lycopersicon esculentum* Mill., and the TSWV-TN isolate was maintained in *Nicotiana tabacum* L. These are the hosts from which these isolates were originally obtained. Other viruses used in serological assays were maintained in appropriate hosts. Polyclonal rabbit antiserum to TSWV was obtained from D. Gonsalves, Cornell University.

Hybridoma production, screening, and isotyping. Hybridomas were produced as previously reported (15), except that medium was supplemented with 2.5 ml/100 ml of ORIGEN (IGEN, Inc., Rockville, MD) for 6 wk after fusion. Hybridomas were screened for antibody production by a modified protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA) (3). Plates (no. 011-010-3350; Dynatech Laboratories, Chantilly, VA) were

first coated with 1 µg/ml of protein-A (Sigma Chemical Co., St. Louis, MO) 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 then a 1/2,000 dilution of anti-TSWV polyclonal antiserum in PBS-Tween was added. After incubation at room temperature for 2 hr, plates were rinsed three times with PBS-Tween, and either TSWV-infected *D. stramonium* or uninfected *D. stramonium*, macerated and diluted 1/100 (w/v) in PBS-Tween with 2% polyvinylpyrrolidone (PVP), was added. After overnight incubation at 4 C, plates were rinsed three times with PBS-Tween, and undiluted culture supernatant was added to plate wells. After a 2.5-hr incubation, the plates were rinsed three times with PBS-Tween, and alkaline phosphatase-linked goat anti-mouse IgG at the manufacturer's recommended working dilution was added (no. A5153; Sigma). After an additional 2.5 hr, the plates were rinsed as above, and p-nitrophenyl phosphate in diethanolamine substrate buffer (1 mg/ml) was added.

Hybridoma cell lines that gave positive reactions only to the TSWV-infected material were cloned by using a soft agar method as previously reported (15). The subclass of immunoglobulin produced by each hybridoma line was determined with a mouse isotype identification kit (Zymed Laboratories, South San Francisco, CA).

MAB was isolated by 50% saturated ammonium sulfate precipitation of culture supernatant of hybridomas grown in HL-1 serum-free medium (Ventrex, Portland, ME). The precipitate was dialyzed against three changes of PBS before use.

ELISA and dot-immunobinding assays (DIBA). To determine the serological reactivity of the MAB to different plant viruses, the modified PAS-ELISA was used as outlined above, except that the polyclonal capture antibody was homologous to the virus being tested. Except for the TSWV capture antibody, which was used at a 1:2,000 dilution, the capture antibodies were used at a 1:1,000 dilution.

Direct double-antibody sandwich ELISA (DAS-ELISA) was conducted according to Clark and Adams (2). Plates were coated for 2 hr with 10 µg/ml of MAB in coating buffer and then rinsed with PBS-Tween. Samples diluted in PBS-Tween containing 2% PVP were added to the wells. After overnight incubation at 4 C, plates were rinsed three times with PBS-Tween, and a 1:400 dilution of alkaline phosphatase (no. P5521; Sigma), conjugated to anti-TSWV MAB, was added. The MAB was conjugated to alkaline phosphatase by using the same procedure outlined by Clark and Adams (2) for the conjugation of polyclonal IgG to the

enzyme. After incubation at room temperature for 4–6 hr, plates were rinsed with PBS-Tween and the substrate added. Plates were incubated and then analyzed with a BIO-TEK EIA plate reader (BIO-TEK Instruments, Inc., Burlington, VT).

PAS-ELISA was carried out as above, except that MAB was used as both the capture and probe antibody at 100 µg/ml, and protein-A alkaline phosphatase (no. P9650; Sigma) at 1 µg/ml in PBS-Tween was used to detect the binding of the probe antibody to the sample.

For the DIBA, tissue samples were prepared by grinding in Tris-buffered saline (TBS) and centrifuging at 6,000 *g* for 10 min. Samples prepared in this manner did not differ in infectivity compared with samples not centrifuged when inoculated to *Petunia hybrida* Vilm., a local-lesion host for TSWV. Samples of 4 µl were spotted on nitrocellulose (NC) sheets and allowed to air dry. Samples on NC sheets were soaked in a 5% solution of Carnation nonfat dry milk for 30 min. NC sheets were then incubated in a 10 µg/ml solution of MAB in TBS for 1 hr. After rinsing in TBS, the NC sheets were incubated for 1 hr in goat anti-mouse alkaline phosphatase (1:1,000 dilution in TBS). The NC sheets were rinsed in TBS and then incubated in a substrate of 5 mg of nitroblue tetrazolium in 15 ml of 0.1 M Tris buffer, pH 9.5, with 0.1 M NaCl and 5 mM MgCl₂ with 2.5 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 50 µl of N,N-diethyl-formamide (12).

To determine the optimal concentration of reagents in both the DAS-ELISA and the PAS-ELISA, the capture and probe antibodies or probe conjugate were added at various concentrations. For the DIBA assay, different incubation times and dilutions of MAB were used to determine satisfactory conditions for the assay.

Identification of proteins reacting to MAB to TSWV. To determine to which of the proteins of TSWV that the MABs produced reacted, the virus was dissociated and the proteins separated according to the method of Verkleij and Peters (16). TSWV was dissociated by the addition of Nonidet-P40 (NP40) to a 1-ml suspension of virus obtained from 50 g of *D. stramonium* to a final concentration of 2%. The viral glycoproteins were separated from the nucleocapsid protein by centrifugation through a sucrose gradient and the proteins concentrated by dialysis (16). The reaction of the MAB to the protein fractions was tested in ELISA and the protein composition of each fraction analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (11,16).

RESULTS

MAB production and characterization. One of the initial 62 hybridomas that tested positive by ELISA for production of antibodies to TSWV had strong reactivity to TSWV in ELISA and did not appear to react with any of the viruses tested (Table 1). Because of the superior performance of MAB from clone 1-7B4, it was used for the remainder of the research. This clone produced immunoglobulin of the IgG2b subclass.

Reaction of TSWV-MAB 1-7B4 in DAS-ELISA, PAS-ELISA, and DIBA. DAS-ELISA was tested because of the widespread use of this assay. PAS-ELISA was tested because antibody does not need to be modified for this assay and because of the affinity of mouse immunoglobulin of the IgG2b subclass to protein-A. The DIBA was tested because the assay can be completed in a short period of time and has been shown to work with MAB to detect nonenveloped viruses (1,15).

For comparison of sensitivity of the three assays in detecting TSWV from different hosts, the samples were prepared as outlined in Materials and Methods, dilutions made as appropriate, and the sample used in each of the three assays. For the DAS-ELISA and PAS-ELISA, a reading was considered positive if the absorbance value at 405 nm was greater than the average absorbance value of the control plus three standard deviations. In the DIBA, a sample was considered positive if a discernible difference in color between the sample and the control was evident on the NC sheet. In either DAS-ELISA or PAS-ELISA, regardless of the isolate and host, TSWV could be detected at a 1/2,560 dilution after 30 min (Table

2). The TSWV-OK and TSWV-TN isolates could be detected at a 1/5,120 dilution, the greatest dilution tried, with either ELISA. In the DIBA, the greatest dilution that could be detected was 1/1,280 with the TSWV-OK isolate in *D. stramonium*. The other isolates were detectable at dilutions of one- or twofold less (Table 2). In addition to the isolates indicated in Table 2, the same assays were conducted on the TSWV-AR isolate maintained in *D. stramonium*. The results were the same as for the TSWV-OK isolate maintained in *D. stramonium*.

Storage of samples for detection of TSWV. The utility of the MAB 1-7B4 in diagnosis was further examined by looking at the reaction of samples stored in various fashions for different lengths of time. Samples of the TSWV-T1 and TSWV-T2 isolates in tomato, and the TSWV-TN in tobacco, were used in this assay. Material was either ground in sample buffer or left intact before storing at 4 C or –20 C for different periods of time. Material ground before storage lost most of its serological reactivity regardless if it was stored at 4 C or –20 C (data not presented). Virus could readily be detected in material left intact and stored at 4 C for 14 days, but much of the serological reactivity of the material stored intact at –20 C was lost (Table 3).

Identification of antigen reacting with TSWV-MAB. A number of attempts were made by western blotting to determine which of the proteins of TSWV served as the homologous antigen for the MAB produced (8). However, when TSWV was denatured with SDS or several other ionic or nonionic detergents before electrophoresis, the serological reactivity of the preparation was lost.

When TSWV was disrupted according to Verkleij and Peters (16), two fractions were obtained. One fraction contained the proteins of approximately 52 kDa, 58 kDa, and 78 kDa, which are the glycoproteins. The other fraction contained only the 29-kDa fraction. When the fractions were run in DAS-ELISA, the MAB 1-7B4 reacted only to the fraction with the 29-kDa protein, indicating that the MAB reacts to the nucleocapsid protein (Table 4).

DISCUSSION

The results presented here indicate that use of the MAB technology is a feasible approach for the detection of the enveloped plant virus TSWV. However, during the initial screening for antibody production by the hybridomas, a sandwich-type assay had to be used. Attempts to bind the virus to the plastic wells with

TABLE 1. Reaction of tomato spotted wilt virus-specific monoclonal antibodies (MAB) to selected viruses in modified protein-A sandwich ELISA^a

Sample ^b	MAB from hybridoma clone 1-7B4 dilution of MAB ^c		
	10 ⁻²	10 ⁻³	10 ⁻⁴
TSWV	0.939	0.617	0.190
PMV	0.016	0.025	0.022
PStV	0.013	0.020	0.026
WMV-1	0.019	0.019	0.037
WSMV	0.021	0.029	0.023
WSBMV	0.016	0.030	0.035
TMV	0.020	0.035	0.037
PVY	0.028	0.030	0.033
CMV	0.000	0.004	0.008
TSV	0.020	0.022	0.035
BMV	0.001	0.020	0.039
Healthy control	0.031	0.021	0.033

^aPlates coated with 1 µg/ml of protein-A. Polyclonal antiserum added at 1:1,000 or 1:2,000 dilution. Samples added at a 1:100 dilution. Samples probed with dilutions of culture supernatant followed by alkaline phosphatase labeled goat anti-mouse antibody.

^bTSWV = tomato spotted wilt virus; PMV = peanut mottle virus; PStV = peanut stripe virus; WMV-1 = watermelon mosaic virus; WSMV = wheat streak mosaic virus; WSBMV = wheat soilborne mosaic virus; TMV = tobacco mosaic virus; PVY = potato virus Y; CMV = cucumber mosaic virus; TSV = tobacco streak virus; BMV = brome mosaic virus.

^cEach value is the average of three replications.

TABLE 2. Reaction of tomato spotted wilt virus (TSWV)-specific monoclonal antibody 1-7B4 in double-antibody sandwich (DAS) ELISA, protein-A sandwich (PAS) ELISA, and a dot-immunobinding assay (DIBA) to isolates of TSWV in different hosts^a

Virus isolate and host	DAS-ELISA			PAS-ELISA			DIBA
	Dilution of virus sample	Absorbance (A_{405} nm)		Dilution of virus sample	Absorbance (A_{405} nm)		Maximum dilution detected ^c
		Virus sample	Healthy control ^b		Virus sample	Healthy control	
TSWV-OK <i>D. stramonium</i>	1/1,280	0.734 ± 0.012	0.006 ± 0.002	1/1,280	0.332 ± 0.015	0.008 ± 0.004	1/1,280
	1/2,560	0.198 ± 0.011	do	1/2,560	0.248 ± 0.023	do	
	1/5,120	0.039 ± 0.007	do	1/5,120	0.051 ± 0.007	do	
TSWV-TN tobacco	1/1,280	0.543 ± 0.021	0.002 ± 0.001	1/1,280	0.335 ± 0.033	0.012 ± 0.004	1/640
	1/2,560	0.157 ± 0.011	do	1/2,560	0.228 ± 0.056	do	
	1/5,120	0.007 ± 0.002	do	1/5,120	0.036 ± 0.009	do	
TSWV-T ₂ tomato	1/1,280	0.057 ± 0.011	0.001 ± 0.001	1/1,280	0.063 ± 0.011	0.003 ± 0.001	1/320
	1/2,560	0.019 ± 0.008	do	1/2,560	0.018 ± 0.008	do	
	1/5,120	0.007 ± 0.002	do	1/5,120	0.004 ± 0.001	do	
TSWV-T ₂ tomato	1/1,280	0.023 ± 0.010	0.003 ± 0.001	1/1,280	0.019 ± 0.003	0.005 ± 0.002	1/320
	1/2,560	0.015 ± 0.005	do	1/2,560	0.012 ± 0.003	do	
	1/5,120	0.003 ± 0.002	do	1/5,120	0.004 ± 0.001	do	

^aSee text for concentration of serological reagents used. Plates read after a 30-min incubation.

^bHealthy control material was diluted 1/10 in ELISA tests.

^cMaximum dilution detected determined by intensity of sample spot compared with control at an equal dilution.

TABLE 3. Reaction of tomato spotted wilt virus (TSWV)-specific monoclonal antibody 1-7B4 in double-antibody sandwich ELISA (A_{405} nm) to TSWV-infected tissue stored at 4 C or -20 C before assay^a

Isolate and host	Days stored before ELISA									
	0		2		5		9		14	
	4 C control	-20 C control	4 C	-20 C						
T1 tomato	0.592	0.326	0.483	0.020	0.397	0.033	0.463	0.017	0.500	0.009
T2 tomato	0.510	0.310	0.511	0.018	0.488	0.025	0.454	0.018	0.510	0.010
Healthy tomato	0.001	0.001	0.002	0.008	0.003	0.005	0.004	0.006	0.003	0.005
TSWV-TN tobacco	0.432	0.347	0.411	0.019	0.376	0.025	0.413	0.027	0.372	0.027
Healthy tobacco	0.004	0.012	0.002	0.001	0.003	0.000	0.004	0.007	0.004	0.005

^aPlates were coated with 10 µg/ml of anti-TSWV MAB. Samples were added at a 1:10 dilution. Samples were probed with a 1:400 dilution of alkaline phosphatase conjugated to anti-TSWV MAB. Values are averages of two experiments.

TABLE 4. Reaction of TSWV-specific MAB 1-7B4 to Nonidet-P40-disrupted TSWV in double-antibody sandwich ELISA (A_{405} nm)^a

Virus proteins present ^b	ELISA value
52 kDa, 58 kDa, 78 kDa	0.004
29 kDa	0.435
Nondisrupted control	0.575
Phosphate-buffered saline	0.002

^aPlates were coated with 10 µg/ml of anti-TSWV. Samples were probed with a 1:400 dilution of alkaline phosphatase conjugated to anti-TSWV MAB.

^bSamples of proteins were obtained from TSWV obtained from 50 g of *D. stramonium*. Nondisrupted control was a 1:1,000 dilution of virus obtained from 50 g of *D. stramonium*.

carbonate coating buffer were not successful because no hybridomas tested positive for antibody production when this assay was used. This may be applicable to other enveloped viruses.

The MAB produced was useful in a variety of assays. The PAS-ELISA and DIBA did not require modification of the MAB. The limit of detection of TSWV was much less for the DIBA than for the PAS-ELISA or DAS-ELISA. The assay of choice would depend on the requirements of the situation. Although similar results were obtained for the detection of TSWV by PAS-ELISA and DAS-ELISA, the results can be significantly altered by how recently the protein-A alkaline phosphatase has been prepared. Using protein-A alkaline phosphatase stored for approximately 1 mo lowered the reactivity of the PAS-ELISA considerably. MAB conjugated to alkaline phosphatase retained high reactivity for

several months. The strong reactivity of the conjugated probe was initially unexpected because our previous experience and another report have shown that the conjugation process may reduce the reactivity of the MAB to the antigen (1,13,15).

All five isolates of TSWV reacted to the MAB, demonstrating that the isolates are serologically related even though they were from different hosts and separate geographical areas. The results of the DAS-ELISA with NP40-disrupted virions indicate that the nucleocapsid protein of TSWV was the homologous antigen for MAB 1-7B4. This protein may be conserved between isolates of the virus just as is the nucleocapsid protein of isolates of many other viruses. It will be interesting to see as more MABs are made to enveloped plant viruses what proteins will serve as useful antigens. Francki and Hatta (4) indicated the serological diagnosis of TSWV has not been adequately addressed. Although polyclonal antiserum of excellent quality can be produced to this virus (5), there are few reports of this being accomplished. The MAB technology has been shown in this paper to have the potential to address the problem of producing significant amounts of antibody for the diagnosis and study of TSWV. This technique should prove to be useful for other enveloped plant viruses.

Note added in proof:

Since this paper has been accepted, a paper on monoclonal antibodies to an enveloped plant virus has been printed. Dietzgen, R. G., and Francki, R. I. B. 1988, Analysis of lettuce necrotic

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