Production of Monoclonal Antibodies to Beet Western Yellows Virus and Potato Leafroll Virus and Their Use in Luteovirus Detection

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ABSTRACT

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Mouse line RBF/Dn immunized with beet western yellows virus (BWYV) resulted in two hybridoma clones secreting BWYV-specific antibodies. Another mouse line (BALB/c) immunized with a combination of BWYV and potato leafroll virus (PLRV) resulted in 17 hybridomas that tested positive for either BWYV or PLRV and one that reacted with an epitope common to both viruses. Selected monoclonal antibodies specific to either BWYV or PLRV were used in ELISA tests to detect luteovirus strains and isolates. Isolates of beet mild yellowing virus, turnip yellows virus, and the RPV (*Rhopalosiphum padi*-transmitted) strain of BYDV were closely related to BWYV, whereas solanum yellows virus and four isolates from potato were identified as PLRV. None of the monoclonals reacted with healthy plant sap.

Potato leafroll is a major disease of potato and is responsible for high yield losses throughout the world wherever potatoes are grown (20). The causal agent was thought to be potato leafroll virus (PLRV), but there is evidence that beet western yellows virus (BWYV) may be part of a virus complex associated with the disease (6). In seed potato certification schemes, potato leafroll disease can be diagnosed on the basis of symptoms induced on potato cultivars, but serology is used for confirmatory tests. Programs that rely on antiserum produced against PLRV may not detect plants infected with BWYV (16,23). In the past, polyclonal antisera prepared against PLRV, BWYV, and other luteoviruses in rabbits often have had relatively high levels of nonspecific reaction (3,18), and those reactions can lead to false-positive results in ELISA (10). Variability in the quality of polyclonal antisera can lead to disagreements among investigators working with the same antigen in different laboratories (11). Often, high-quality reference or diagnostic polyclonal antisera are not available to every investigator.

Hybridoma technology, introduced by Köhler and Milstein (14), revolutionized antibody production and eliminated many problems associated with polyclonal antiserum. This technology provides a means to produce an unlimited, uniform supply of antibody of a required specificity (8). The objective of this research was to produce monoclonal antibodies for detection and differentiation of BWYV and PLRV.

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MATERIALS AND METHODS

Virus isolates and purification. The PLRV strain used for purification was the one characterized by Rowhani and Stace-Smith (22) and is designated herein PLRV-BC. PLRV-BC was propagated in ground cherry (*Physalis pubescens* L.) inoculated by viruliferous green peach aphids (*Myzus persicae* (Sulzer)) in a growth chamber (21 C, 105 $\mu E \cdot m^{-2} \cdot s^{-1}$ fluorescent and incandescent light) with a 72-hr access period. The aphids were killed by spraying the plants with pirimicarb (0.25 g a.i./L) and the plants were moved to a greenhouse (15–25 C) for 4–6 wk. Leaf tissue was harvested and used immediately or frozen at −80 C until used. A sugar beet isolate of BWYV from British Columbia (15) was used for virus purification and will be referred to as BWYV-BC. *P. pubescens* was inoculated with BWYV-BC and propagated in a separate growth chamber and greenhouse with the same procedure as for PLRV-BC.

Both PLRV and BWYV were purified by the method of D'Arcy et al (4). The virus concentration was estimated from the UV-absorption spectrum assuming an extinction coefficient of $A_{260}^{0.1\%} = 8.6$ based on the value calculated for PLRV by Takanami and Kubo (25).

Other luteovirus isolates. Sources of luteoviruses include J. E. Duffus (Salinas, CA), G. Johnstone (Hobart, Tasmania), C. D'Arcy (Urbana, IL), O. Gutbrod (Corvallis, OR), R. Clarke (Idaho Falls, ID), B. Falk (Davis, CA), and N. S. Wright (Vancouver, BC). Some of these isolates have been previously identified as being serologically related but not necessarily identical to BWYV or PLRV. A total of 23 luteovirus strains and isolates were tested by ELISA using monoclonal antibodies against PLRV and BWYV.

Antibodies. Rabbit antiserum against PLRV was obtained from R. Stace-Smith (Agriculture Canada Research Station, Vancouver, British Columbia). A rabbit antiserum against a Californian isolate of BWYV from broccoli (RY-1-R) was a gift from J. E. Duffus (USDA-ARS, Salinas, CA). A monoclonal antibody (VRS1), specific for PLRV, was a gift from R. Martin (18). Immunoglobulins were purified from antisera by ammonium sulfate precipitation and DEAE-cellulose column chromatography as described by Clark et al (2). The immunoglobulins were adjusted to approximately 1 mg/ml ($A_{280} = 1.4$) and stored in aliquots of 0.5 ml at −20 C.

Hybridoma production, screening, and isotyping. Two immunization procedures were used. In the first, RBF/Dn mice (Jackson Laboratories, Inc., Bar Harbour, ME) were immunized with three injections of purified BWYV in 0.05 M phosphate buffer, pH 7.0. The first injection, 50 μg of BWYV emulsified with Freund's incomplete adjuvant, was given subcutaneously. The second and third injections of 50 μg each were administered intraperitoneally at 4 and 6 wk after the first injection. Three days after the third injection, the mice were killed by CO_2 asphyxiation and the spleens were removed aseptically. In the second procedure, BALB/c mice were immunized with three injections of virus. The first injection was 50 μg each of purified BWYV and PLRV emulsified with Freund's incomplete adjuvant and given subcutaneously. The second injection was 50 μg of PLRV given 4 wk later intraperitoneally in 0.05 M phosphate buffer, pH 7.0. The third and final booster was 50 μg of BWYV given intraperitoneally in phosphate buffer. Three days later, the mice were sacrificed and the spleens were harvested as described earlier.

The same fusion protocol was used for both immunization procedures. FOX-NY myeloma cells (Hyclone Laboratories, Inc., Logan, UT) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1 mM of pyruvate and 2 mM of L-glutamine and 10% fetal calf serum (FCS). Spleen cells were fused with myeloma cells (spleen cell/myeloma cell ratio approximately 10:1) in 50% polyethylene glycol 4000 as described by Kannangara et al (13). All myelomas and hybridomas were grown at 37 C in an atmosphere of 10% CO_2.

The cell fusion mixture was dispensed into five 96-well culture plates and incubated overnight in nonselective media (DMEM containing 20% FCS) with mouse thymocytes as feeder cells. The microcultures were then fed with AAT selection media (7.5×10^{-5} M adenine, 8×10^{-7} M aminopterin, and 1.6×10^{-5} M thymidine [24]). After 10 days, culture fluids from the hybridomas were screened for anti-BWYV and anti-PLRV antibody production by an indirect triple-antibody sandwich ELISA (TAS-ELISA) described in detail below. Culture fluid from the hybridomas was also screened for antibodies against healthy plant sap. Hybridoma cell lines that produced antibodies that tested positively with either BWYV or PLRV and negatively against healthy potato and ground cherry sap were cloned twice by limiting dilution, grown in cell culture, retested, and the positive cultures were stored under liquid nitrogen.

For production of antibodies in ascitic fluid, BALB/c mice were primed intraperitoneally with pristane 10 days before intraperitoneal injection of approximately 10^7 hybridoma cells. Some BALB/c mice were also immunosuppressed by injection of cyclophosphamide (0.025 g/g of animal weight) 24 hr before injection of the hybridoma cells. Ascitic fluid was collected 10–20 days later by inserting an 18-gauge needle into the peritoneal cavity of mice showing pronounced abdominal swelling. After low-speed centrifugation at 8,700 *g* for 20 min to remove cellular debris, the ascitic fluid supernatant was mixed with an equal volume of saturated ammonium sulfate and stored at 4 C until required.

The monoclonal antibody isotypes were determined by double-antibody sandwich ELISA (DAS-ELISA) using a mouse hybridoma subisotyping kit (Calbiochem, Behring Diagnostics, La Jolla, CA). Subcloned hybridoma supernatant fluids were tested using the manufacturer's protocol.

ELISA. An indirect TAS-ELISA procedure was used for screening hybridomas for antibody production and for testing antibody specificity (18). All reagents were used at 100 µl per well in flat-bottomed Lindbro microtiter plates (Flow Laboratories, Mississauga, Ontario) except the blocking steps, which

Table 1. Characteristics of monoclonal antibodies produced by immunization of RBF/Dn or BALB/c mice with beet western yellows virus (BWYV) alone or in combination with potato leafroll virus (PLRV)

Mouse line	Monoclonal antibody	Subclass	Immunogen	Homologous antigen
RBF/Dn	510H	IgG2a	BWYV	BWYV
RBF/Dn	112E	IgG1	BWYV	BWYV
BALB/c	13CD	IgM	BWYV + PLRV	BWYV
BALB/c	15CD	IgM	BWYV + PLRV	BWYV
BALB/c	31CC	IgM	BWYV + PLRV	BWYV
BALB/c	43GB	IgM	BWYV + PLRV	BWYV
BALB/c	26BE	IgG1	BWYV + PLRV	PLRV
BALB/c	41BC	IgM	BWYV + PLRV	PLRV
BALB/c	43BC	IgM	BWYV + PLRV	BWYV + PLRV

Table 2. Reactions[a] of 23 luteovirus strains or isolates in triple-antibody sandwich ELISA tests using six monoclonal antibodies specific to beet western yellows virus (BWYV), three specific to potato leafroll virus (PLRV), and one that reacts against both BWYV and PLRV

	Monoclonal antibodies									
	BWYV-specific						PLRV-specific			BWYV + PLRV specific
Luteovirus strain or isolate	510H	112E	13CD	15CD	31CC	43GB	26BE	41BC	VRS1	43BC
Beet western yellows virus										
BWYV-BC[b]	+++	++	++	+	++	++	−	−	−	+++
BWYV-RY-1-R[c]	+++	+++	+++	+	++	++	−	−	−	+++
BWYV-RY-7[c]	+++	+++	+++	+++	++	++	−	−	−	+++
BWYV-SAL(9)[c]	+++	+++	+++	+++	+++	+++	−	−	−	+++
BWYV-NETH[c]	+++	+++	+++	+	+++	+++	−	−	−	+++
BWYV-150[c]	++	++	++	+	+	+	−	−	−	++
BWYV-B10[c]	+	+	++	+	+	+	−	−	−	+
BWYV-RB2[c]	+++	+++	+++	+	++	+++	−	−	−	+++
BWYV-MP4BF[c]	++	++	+	+	+	+	−	−	−	+
BWYV-MY4[c]	++	+	+	+	+	++	−	−	−	+
BMYV-324[c]	+++	++	+	+	+	+	−	−	−	+
TuMV[c]	+++	+++	+++	+	+++	+++	−	−	−	+++
Barley yellow dwarf virus										
BYDV-RPV-NY[d]	++	++	−	−	−	−	−	−	−	−
BYDV-MAV-NY[d]	−	−	−	−	−	−	−	−	−	−
BYDV-PAV-IL[e]	−	−	−	−	−	−	−	−	−	−
Potato leafroll virus										
PLRV-BC[b]	−	−	−	−	−	−	++	+++	+++	+
PLRV-ST4[b]	−	−	−	−	−	−	++	+++	+++	+
PLRV-ORE[f]	−	−	−	−	−	−	++	+++	+++	+
PLRV-ID[g]	−	−	−	−	−	−	+++	+++	+++	+
Solanum yellows virus										
SYV-21[c]	−	−	−	−	−	−	+	+	+	+
SYV-44[c]	−	−	−	−	−	−	++	++	++	+
Soybean dwarf virus										
SDV-AS[h]	−	−	−	−	−	−	−	−	−	−
SDV-AP[h]	−	−	−	−	−	−	−	−	−	−

[a] Reactions (A_{405}) after overnight incubation of substrate: +++ >1.0; ++ = 0.5–1.0; + <0.5; and − = negative.
[b] N. S. Wright, Agriculture Canada, Vancouver Research Station, Vancouver, British Columbia, Canada.
[c] J. E. Duffus, USDA-ARS, Salinas, CA.
[d] B. Falk, University of California, Davis, CA.
[e] C. D'Arcy, University of Illinois, Urbana, IL.
[f] O. Gutbrod, Oregon State University, Corvallis, OR.
[g] R. Clarke, Idaho Crop Improvement Association, Idaho Falls, ID.
[h] G. Johnstone, Tasmania Department of Agriculture, Hobart, Australia.

were 300 µl per well. Plates were coated overnight at 4 C with serum immunoglobulins homologous to the antigen to be tested and diluted to 1 µg/ml in phosphate-buffered saline (PBS) (127 mM NaCl, 2.6 mM KCl, 8.5 mM Na_2HPO_4, and 1.1 mM KH_2PO_4). Plates were blocked with 0.2% nonfat milk powder in PBS (w/v) for 30 min at room temperature (12). Leaf tissue (0.1 g) from infected and healthy plants was ground in a sap extractor (Erich Pollähne, Wennigsen, Germany), with 0.9 ml of extraction buffer (PBS containing 0.05% Tween 20, 2% PVP, and 0.2% nonfat milk powder) added by drops onto the beveled rollers. Isolates provided by J. E. Duffus as freeze-dried cultures were ground with a mortar and pestle (0.02 g/ml of extraction buffer). Extracts were incubated in the wells overnight at 4 C. Plates were then washed three times for 20 s with PBS containing 0.05% Tween 20 (PBS-T) and then blocked again as described earlier. Homologous or heterologous monoclonal antibodies, from culture fluid supernatants, were diluted 1:1 in PBS-T containing 0.2% nonfat milk powder (PBS-T-M) and incubated in the wells for 3 hr at room temperature. Plates were washed as above and conjugate, rabbit anti-mouse alkaline phosphatase (Jackson Immunoresearch Laboratories, Inc., Avondale, PA), diluted 1:5,000 in PBS-T-M, was incubated in the wells for 3 hr at room temperature. After washing the plates as described earlier, substrate (*p*-nitrophenyl phosphate) at 0.5 mg/ml in 10% diethanolamine, pH 9.8, was added to the wells and incubated for 2 hr at room temperature and then overnight at room temperature.

The absorbance of each well was measured at A_{405} in a Titertek Multiscan MCC plate reader (Flow Laboratories). ELISA reactions were scored +++ for measurements of >1.0, ++ for measurements of 0.5–1.0, and + for measurements of <0.5 but greater than three times the mean of the measurements of the healthy control plants. Values below this threshold were scored − for negative.

RESULTS

Production of hybridomas. From the first immunization protocol and fusion, where BWYV was used as the immunogen, two hybridoma clones that secreted BWYV-specific antibodies were obtained. Clones 510H and 112E produced ascitic fluids in BALB/c mice, after immunosuppression with cyclophosphamide, with titers of 10^{-8} and 10^{-4}, respectively, in TAS-ELISA. Clone 510H produced between 3 and 8 ml of ascitic fluid per mouse, whereas clone 112E tended to produce solid tumors and less than 1 ml of ascites per mouse.

The fusion from the second immunization protocol, where both BWYV and PLRV were injected, resulted in 18 of 420 wells with hybridomas that tested positive for production of antibody to either BWYV or PLRV, or both, but not to healthy plant sap from potato or ground cherry. Seven of these hybridomas were successfully cloned and the subclasses of the monoclonal antibodies were determined. Of the seven monoclonals, three were specific to BWYV, two to PLRV, and one reacted to both BWYV and PLRV (Table 1).

Reaction of luteovirus strains and isolates. Thirteen of the 23 luteovirus strains or isolates were BWYV or closely related serologically to BWYV (Table 2). BWYV includes the isolates identified by the synonyms BMYV (9), TuYV (7), and the strain of barley yellow dwarf virus transmitted by *Rhopalosiphum padi* L. (BYDV-RPV) (1,17,21). Two monoclonal antibodies, 510H and 112E, reacted with all of the BWYV strains or isolates tested and BYDV-RPV. Four others (13CD, 15CD, 31CC, and 43GB) reacted with all of the BWYV strains and isolates except BYDV-RPV. All of the monoclonals that reacted with the PLRV isolates (26BE, 41BC, and VRS1) also reacted with the two isolates of solanum yellows virus (SYV) (19). One monoclonal antibody (43BC) reacted with an epitope common to BWYV, PLRV, and SYV. None of the monoclonal antibodies reacted with the other luteoviruses nor with healthy plant sap.

DISCUSSION

Although the serological relationships among the luteoviruses are complex (26), monoclonal antibodies provide a tool to discriminate among them. Monoclonal antibodies 510H and 112E reacted with all of the BWYV strains and isolates tested, including BYDV-RPV, which is now considered to be strain of BWYV (1,5,17). Four other monoclonals detected all of the BWYV strains except BYDV-RPV. Any of these six monoclonals could be used for screening plant tissue for BWYV in dicotyledonous species. Monoclonal antibody 510H is the most suitable for large-scale testing because of its broad specificity for BWYV, detecting 13 isolates from five countries, and because of its high titer in ascitic fluid.

Solanum yellows virus was described as an apparently distinct member of the luteovirus group, based on its affinity to the Solanaceae and its serological relatedness to BWYV and BYDV-RPV (19). The virus was listed as a probable strain or synonym of PLRV in a recent description of the luteovirus group (26). The work reported in this paper clearly showed that the virus is serologically closely related to PLRV and, therefore, should be considered a synonym or strain of PLRV. Monoclonals 26BE, 41BC, and 43BC reacted with all the PLRV strains and isolates and both isolates of SYV tested. None of the monoclonals detected either strain of SDV or the MAV and PAV strains of BYDV. The specificity of monoclonal antibodies 26BC and 41BC appeared to be the same as monoclonal antibody VRS1 produced by Martin and Stace-Smith (18).

The monoclonal antibodies described here can be used effectively in a routine diagnostic TAS-ELISA for the detection and identification of BWYV and PLRV in plant tissue. This paper does not address the question whether BWYV is an important component of the potato leafroll disease complex (6). This aspect is being investigated and will be reported on later.

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