A Monoclonal Antibody That Discriminates Strains of Citrus Tristeza Virus

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ABSTRACT

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A monoclonal antibody produced to the Florida citrus tristeza virus (CTV) isolate T-36 (CTV-MCA13) reacted to decline-inducing, seedling yellows, and stem-pitting isolates of CTV from Florida, California, and Spain. It did not react to CTV isolates from these same areas, which produce symptoms primarily in Mexican lime. All CTV antigen sources used reacted strongly to anti-CTV polyclonal antibodies in double antibody sandwich enzyme-linked immunosorbent assays (ELISA) and to a previously reported broad spectrum CTV monoclonal antibody (3DF1) in comparable double antibody sandwich indirect ELISA. Discrimination of CTV isolates by CTV-MCA13 was similar in indirect ELISA with plate-trapped antigen and in double antibody sandwich indirect ELISA with antigen trapped on polyclonal antibody-coated plates. Results of immunoelectron microscopy suggest that CTV-MCA13 may react to a cryptic epitope on the viral coat protein. The monoclonal antibody is an IgG2a immunoglobulin and did not react to extracts of healthy citrus or citrus infected with other viruses.

Additional keyword: immunoassay.

Citrus tristeza virus (CTV) is the cause of severe stem pitting and decline diseases of citrus and one of the most economically important citrus pathogens worldwide (4,13). There is great diversity of symptoms induced by different isolates of CTV, and symptom severity is often host specific (12). Currently, severity of a given isolate can be determined only by a time-consuming procedure of inoculating differential indicator plants or a commercial host (12). Other techniques for discriminating CTV isolates, such as cDNA probes (23) and dsRNA analysis (8), so far lack the desired specificity or reliability, or are not adaptable for rapid, large-scale assays. There is a need for a rapid diagnostic procedure to identify specific severe isolates of CTV.

Serological tests for CTV have been developed (5,11), and enzyme-linked immunosorbent assays (ELISA) have been used widely for survey, certification, and eradication work, as well as for research (6,10,17). Polyclonal antisera have been made to different isolates in several animal species (10), and a monoclonal antibody has been produced and made available commercially (25). The polyclonal and monoclonal antibodies reported all have reacted to a wide range of CTV isolates of differing symptom severity (14,25). These immunological probes are highly useful for general detection of CTV infection but do not provide information about biological severity. The lack of evidence for serological diversity among CTV isolates was consistent with analysis of peptide digests of different isolates, which also indicated only small differences in coat protein chemistry among the isolates evaluated (19). Brlansky et al (5) and Vela et al (26) have suggested that multiple epitopes exist in the CTV coat protein based on results obtained, respectively, with different polyclonal antisera and with different monoclonal antibodies. Vela et al (26) also suggested that the epitopes identified so far were common to all CTV isolates tested. However, evidence that some epitopes may be strain specific was obtained with polyclonal antiserum to CTV isolate T-36 that showed greater avidity to homologous antigens than to heterologous antigens, whereas antiserum to CTV isolate T-4 reacted equally with both antigens (Garnsey, *unpublished*). In this paper we report production of a monoclonal antibody to the T-36 isolate of CTV (CTV-MCA13) that reacted to T-36 and other isolates of CTV associated with decline, seedling yellows, and stem pitting diseases of citrus.

MATERIALS AND METHODS

Virus isolates. Isolates of CTV used in this study were maintained in glasshouse-grown citrus plants, usually sweet orange (*Citrus sinensis* (L.) Osb.) or Mexican lime (*C. aurantiifolia* (Christm.) Swing.). Isolates foreign to the United States were maintained in a quarantine glasshouse at Beltsville, MD (12). Isolates of CTV ranged in symptom severity from those that produced mild symptoms in Mexican lime and essentially no symptoms in other hosts to those that produced stunting or decline in grafted combinations of sweet orange on sour orange (*C. aurantium* L.), seedling yellows in sour orange seedlings, and stem pitting in grapefruit (*C. paradisi* Macfad.) and/or sweet orange seedlings (4,12). The severity of reactions of these five indicator hosts (12) to 13 isolates of CTV is summarized in Table 1. This information was derived from comparative biological assays at Beltsville (12) and other published reports (1,2,23). These isolates were free of other viruses or viruslike pathogens based on indexing tests and/or aphid transmission of CTV to virus-free citrus seedlings before use in these studies.

Isolates of CTV used for production of the various antisera in this study were T-4, T-36, and T308. Isolate T-36, which was used to produce the discriminating antibody described herein, causes a moderately strong reaction in Mexican lime, a moderate decline in sweet-sour trees, a moderate seedling yellows reaction in sour orange seedlings, and slight stem pitting in grapefruit and sweet orange (23). It is transmissible by *Aphis gossypii* Glov.

(27). The T-26 and T-4 isolates used for preliminary screening of hybridomas produce mild to moderate vein clearing or stem pitting symptoms in Mexican lime only (11).

The exocortis viroid (E-16B), citrus leaf rugose virus (CLRV-2), citrus variegation virus (CVV-2), and tatterleaf-citrange stunt virus (TLCSV-4) sources used in tests to verify specificity were standard, well-characterized, glasshouse sources free of CTV. Sources of healthy citrus tissue used in this study were glasshouse-grown, virus-free plants.

Preparation of plant extracts. Unless specifically noted, tissue used for antigen preparation was bark from shoots of new growth collected when virus titer was expected to be near optimum (11). Extracts from CTV-infected and healthy plants were used to screen hybridomas and to conduct specificity assays. Crude extracts were used for screening and evaluation because of the difficulty in preparing highly purified virus in sufficient quantity from a large number of sources. Fresh extracts were prepared by grinding coarsely chopped tissue in 0.05 M Tris buffer, pH 7.8, with a dispersion homogenizer. The extracts were filtered through cheesecloth to remove debris, stored on ice, and used within 1 day of preparation. For applications requiring a consistent source of antigen, uniform, freeze-dried extracts were prepared by powdering coarsely chopped tissue in liquid nitrogen with a mortar and pestle. Three volumes of cold 0.05 M Tris buffer, pH 7.8, which contained 50 mg/ml of sucrose, was added to the ice powder, and the mixture was extracted by a dispersion homogenizer. The extract was filtered through cheesecloth, frozen on dry ice, and lyophilized in 1- or 2-ml aliquots with a freeze dryer equipped with a shelf freezer and a stoppering device. Lyophilization was for 4 hr with gradually increasing shelf temperature (−25 to +6 C). Freeze-dried extracts were rehydrated to the desired volume in distilled water and used the same day.

Relative concentration of viral antigens in CTV-infected plant tissue was estimated by double antibody sandwich (DAS) ELISA (3) using a purified polyclonal antibody (879) and a dilution series of each antigen source. Based on the reaction curves, dilutions were determined that would give reaction of A_{405} 0.8 to 1.0 with the polyclonal antibody. These dilution values were used in all subsequent tests.

Production of monoclonal antibodies. Balb/c mice were immunized initially by intraperitoneal injection with 100 μg of purified T-36 CTV (20) in Freund's complete adjuvant. These mice were hyperimmunized by intravenous injections with 50 μg of purified T-36 in 0.2 ml of 0.1 M phosphate buffered saline (PBS), pH 7.4, at 1 and 10 mo after the initial immunization. Three days after the final injection, spleen cells were harvested and fused with Sp2/0-Ag-14 myeloma cells using the technique of Van Duesen and Whetstone (24). Blood removed from the hyperimmunized mouse by cardiac puncture was saved as a positive control.

Two weeks after plating, primary hybridomas were screened by indirect ELISA using plate-trapped antigens (see subsection on immunoassays). Selected positive hybridomas were cloned twice by limiting dilution. Antibodies were produced by intraperitoneal injection of pristane-primed mice with approximately 10^6 cells/mouse. The resulting ascites fluid was centrifuged for 10 min at 1,000 *g* with Sure-Sep II serum-plasma separators (General Diagnostics Division, Warner-Lambert Co., Morris Plains, NJ) and filtered. Aliquots were either frozen or purified by affinity chromatography.

Isotype determination. Antibody class and subclass were determined by indirect ELISA using reagents supplied in an isotyping kit (Zymed Laboratory, San Francisco, CA).

Purification of immunoglobulins. Immunoglobulins were purified from ascites fluid using a Beckman rProtein A IgG purification kit (Smithkline Beckman, Fullerton, CA). The IgG from polyclonal antisera were purified by column chromatography on Sephacel DEAE cellulose (Pharmacia Fine Chemicals, Uppsala, Sweden) (3). Concentrations were calculated spectrophotometrically assuming an A_{280} of 1.40 for 1 mg/ml concentrations.

Immunoassays. Two types of indirect ELISA were used in this study. In the first, designated plate-trapped antigens (PTA) were allowed to bind directly to Immulon II plates (Dynatech Laboratories, Inc., Alexandria, VA) for 1 hr at 37 C. Antigens were normally in 0.05 M Tris buffer, pH 7.8, or in PBS. After this step and all succeeding ones, plates were washed three times with 0.1 M PBS-0.05% Tween 20, pH 7.4. After incubation with the antigen preparation, plates were incubated with 1% bovine serum albumin (BSA) in PBS overnight at 4 C. Twenty-five to 50 μl/well of undiluted hybridoma growth medium was added for screening assays, whereas 50 μl/well of 1:2,000 diluted ascites fluid was added for all other assays and incubated for 1 hr at 37 C. After a 1-hr block step with 1% BSA, the plates were incubated with 1:1,000 diluted alkaline phosphatase-labeled goat antimurine IgG or IgM (Sigma Chemical Co., St. Louis, MO) for 1 hr at 37 C. Freshly prepared substrate solution (*p*-nitrophenyl phosphate, Sigma Chemical Co.) was added for 1 hr at 37 C. Reactions were read on an EL 309 Microplate Autoreader (Bio-Tek Instruments, Inc., Burlington, VT) at 405 nm.

The second indirect assay was a DAS-ELISA where plates were coated with polyclonal antibody (1 μg/ml) for 1 hr at room

TABLE 1. Symptom severity of 13 citrus tristeza virus (CTV) isolates in five citrus indicators[a]

CTV isolate		Host plant						
Designation	Origin[a]	Mexican lime	Sweet on sour	Sour orange seedling	Grapefruit	Madam Vinous sweet orange	Profile[c] score	Cumulative score[d]
SY-568	Ca	3[b]	3	3	2	3	3:6:9:8:15	41
SY-576	Ca	3	3	2	2	1	3:6:6:8:5	28
T-388	Sp	3	3	2	2	1	3:6:6:8:5	28
T-68	Fl	2	3	2	1	0	2:6:6:4:0	18
T-36	Fl	2	2	2	0.5	0	2:4:6:2:0	14
T-66a	Fl	2	2	0.5	0	0	2:4:2:0:0	8
T-514	Ca	2	0.5	0	0.5	0	2:1:0:2:0	5
T-300	Sp	2	0.5	0	0	0	2:1:0:0:0	3
T-4	Fl	2	0	0	0	0	2:0:0:0:0	2
T-516	Ca	1	0.5	0	0	0	1:1:0:0:0	2
T-26	Fl	1	0	0	0	0	1:0:0:0:0	1
T-30	Fl	1	0	0	0	0	1:0:0:0:0	1
T-385	Sp	1	0	0	0	0	1:0:0:0:0	1

[a] Fl = Florida, Ca = California, Sp = Spain.

[b] Symptom severity rated on a scale of 0 to 3, with 3 being the most severe.

[c] Score of the host reaction multiplied by a weighing factor of 1 for Mexican lime symptoms, 2 for stunting in grafted combinations of sweet orange on sour orange, 3 for seedling yellows in sour orange seedlings, 4 for seedling yellows and stem pitting in grapefruit seedlings, and 5 for stem pitting and stunting in Madam Vinous sweet orange gives weighted profile score (12).

[d] Sum of profile scores.

temperature using conventional ELISA protocols previously described (3). The polyclonal antisera used were antisera to unfixed, purified CTV isolates T-4 (879 antiserum) or T-36 (1052 antiserum) (15). Both sera reacted specifically to CTV in conventional DAS-ELISA tests. Antiserum 1052 reacted more strongly with its homologous antigen than with heterologous CTV isolates (Garnsey et al, *unpublished*). The monoclonal antibody 3DF1, previously reported reactive to all CTV strains tested (25,26), was used as the reference antibody in DAS indirect assays. Purified 3DF1 was generously provided by C. Vela and M. Cambra.

Immunoelectron microscopy. Two techniques were used to verify MCA13 binding to CTV virus. The first was a serum-specific electron microscopy (SSEM) procedure (5) where carbon-coated, 300-mesh copper grids were coated with MCA13 or purified 879 antiserum, floated on CTV-infected plant extracts, then stained with uranyl acetate, pH 3.0. The second technique was an immunogold-labeling procedure. Grids were coated with 879 antiserum and floated on CTV-infected extracts as in SSEM. The grids then were washed by floating on drops of PBS three times for 5 min, followed by floating for 3 hr on diluted 1:1,000 CTV-MCA13 ascites. Grids were washed as above and floated on 20 nm of gold-labeled goat antimouse IgG (E-Y Laboratories, San Mateo, CA) for 2 hr. Washing was repeated and grids were stained with uranyl acetate as in the SSEM procedure. Immunogold-labeled control grids were processed as above, except that the CTV-MCA13 coating step was eliminated. The grids were examined by a Phillips 201 transmission-electron microscope for CTV detection and gold labeling.

RESULTS

Production of monoclonal antibodies. The fusion of spleen and myeloma cells yielded 960 wells with actively growing primary hybridomas. Media from each well were tested against extracts from healthy and T-36-infected plant tissue in indirect ELISA. One hundred and forty wells contained primary hybridomas that produced antibodies that reacted specifically with T-36-infected tissue. These 140 primary hybridomas were retested with extracts from healthy tissue and tissue infected with CTV isolates T-4, T-26, and T-36. Primary hybridomas in the second screening assay were separated into five groups: those that reacted: 1) only to T-36-infected tissue; 2) with all CTV-infected tissue in the test; 3) to T-36- and T-26-infected tissue more than to T-4-infected tissue; 4) to T-36- and T-4-infected tissue more than to T-26-infected tissue; and 5) to T-26-infected tissue to a greater extent than to T-36- and T-4-infected plant tissue. The ability to classify primary hybridomas in five reaction categories suggested serological diversity among isolates tested.

The specificity of one cell line that reacted only to T-36 is further described here. We have designated this cell line CTV-MCA13. This cell line was cloned twice, and ascites fluid, containing antibody of the IgG2a subclass, was produced.

Immunoassays. Extracts from citrus plants infected with 13 different CTV isolates, selected to represent a wide range of biological severity and three different geographic areas, were assayed. Dilution factors calculated for each extract source to yield an approximately uniform reaction in the polyclonal DAS-ELISA reference test ranged from 1/40 to 1/800. These extracts were tested in a DAS indirect assay against CTV-MCA13 and against the CTV broad spectrum monoclonal antibody 3DF1 (26). Reactions greater than four times the healthy control reaction were considered positive. All 13 extracts reacted positively to 3DF1, and all but two A_{405} values ranged from 0.86 to 1.16 (Table 2). In contrast, only six of the 13 extracts reacted positively to the CTV-MCA13 antibody (Table 2) with A_{405} values from 0.34 to 0.87. These six isolates all have been associated with decline in sweet orange grafted on sour orange, seedling yellows, and/or stem pitting in sweet orange or grapefruit (Table 1). The isolates that did not react to CTV-MCA13 cause appreciable symptoms in Mexican lime only.

A similar discrimination of CTV isolates by CTV-MCA13 antibody was observed in PTA indirect ELISA tests with the same extracts (Fig. 1). Figure 1 also shows a correlation between biological (standard host range test cumulative score) and CTV-MCA13-based serological (PTA indirect ELISA) assays for CTV symptom severity. Although specificity of CTV-MCA13 was not affected by the type of ELISA used, reaction of CTV-MCA13, relative to 3DF1 monoclonal antibody, was stronger in PTA indirect ELISA than in the DAS indirect ELISA (data not shown). ELISA data presented in Table 2 and Figure 1 are the means of 12 replications. No reaction was observed to extracts from citrus plants infected with three citrus viruses unrelated to CTV (CVV, CLRV, and TLCSV) and with citrus exocortis viroid.

Immunoelectron microscopy. Results of immunoelectron microscopy indicated that CTV-MCA13 did not bind to whole virus particles from CTV isolate T-36-infected plant tissue. SSEM micrographs of grids coated with purified 879 antiserum showed large numbers of trapped virus particles, whereas micrographs of grids coated with CTV-MCA13 showed no trapped virus. In the immunogold-labeling procedure, grids coated with polyclonal 879 anti-CTV antibody first, followed by CTV-infected plant tissue, CTV-MCA13, and an antimouse gold-labeled antibody were compared to control grids not treated with CTV-MCA13. Both treatments showed large numbers of trapped virus particles, but gold labeling was less in the control grids. The gold particles appeared to be randomly scattered throughout the grid and not bound to whole virus particles.

TABLE 2. Reactivity of citrus tristeza virus (CTV)-monoclonal antibody (MCA) 13 and the commercially available 3DF1 monoclonal antibodies to 13 CTV isolates in double antibody sandwich (DAS) enzyme-linked immunosorbent assay (ELISA)

Antigen designation	Cumulative[a]	DAS indirect ELISA (A_{405} mean ± standard deviation) CTV-MCA13	3DF1
SY-568	41	0.79 ± 0.04	1.16 ± 0.03
SY-576	28	0.34 ± 0.00	0.53 ± 0.01
T-388	28	0.47 ± 0.01	0.90 ± 0.04
T-68	18	0.60 ± 0.03	1.02 ± 0.03
T-36	14	0.78 ± 0.06	1.01 ± 0.02
T-66a	8	0.87 ± 0.10	1.04 ± 0.01
T-514	5	0.09 ± 0.00	0.54 ± 0.01
T-300	3	0.07 ± 0.01	1.05 ± 0.03
T-516	2	0.11 ± 0.00	0.86 ± 0.02
T-4	2	0.06 ± 0.00	1.03 ± 0.04
T-385	1	0.07 ± 0.01	1.08 ± 0.04
T-30	1	0.06 ± 0.00	0.96 ± 0.06
T-26	1	0.05 ± 0.00	0.93 ± 0.03
Healthy	NA[b]	0.06 ± 0.02	0.05 ± 0.01

[a] Cumultive score from Table 1.
[b] NA = Not applicable.

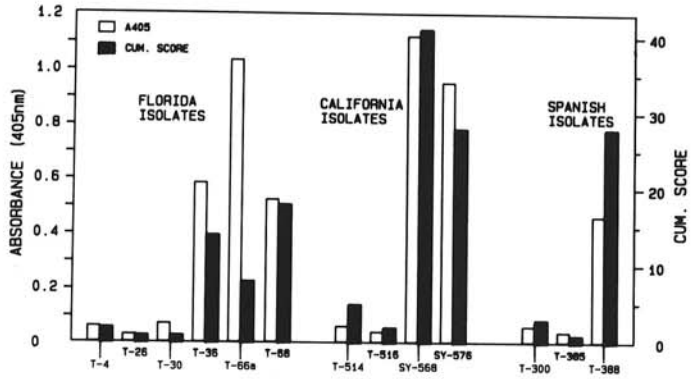

Fig. 1. Comparison of biological and serological assays for citrus tristeza virus (CTV) symptom severity. Black bars represent standard host range test (12) cumulative scores from Table 1. White bars represent CTV-MCA13-based plate-trapped antigen indirect enzyme-linked immunosorbent assay reactivity (A_{405}).

DISCUSSION

The results presented clearly indicate that the CTV-MCA13 monoclonal antibody prepared to CTV isolate T-36 reacts differentially to the CTV isolates tested. Previous work by Vela et al (25) and Gumpf et al (16) and the demonstration of quantitative differences in reaction among specific antibody-antigen combinations by Garnsey (*unpublished*) had suggested that some antigenic variability could exist among isolates of CTV. This report, however, provides the first direct evidence for a specific epitope which occurs in some CTV isolates and is apparently absent in others.

The exact nature and location of the epitope is unknown at present, but the lack of gold labeling of intact virus particles in immunoelectron microscopy and the high activity of the antibody against PTA relative to DAS-ELISA suggest that it may be a cryptic epitope (9). An assumption is made that virus extracts contain both whole and partially degraded virus and that the polyclonal antiserum contains antibodies that will bind to cryptic epitopes exposed on those degraded virus particles.

The CTV-MCA13 antibody reacted with the immunogen, T-36, and five of the 12 other isolates tested (Table 2 and Fig. 1). Most, if not all, of these five isolates have biological properties distinct from T-36. All produce decline or stunting in trees grafted on sour orange rootstocks and/or stem pitting in grapefruit or sweet orange. The seven isolates that did not react to CTV-MCA13 cause symptoms in Mexican lime but do not cause significant stunting in sweet orange grafted on sour orange, seedling yellows, or stem pitting in grapefruit or sweet orange under greenhouse conditions. Preliminary tests with a broader range of CTV isolates have confirmed this pattern (data not shown).

The specificity of CTV-MCA13 presumably is correlated with a specific property of the virion coat protein. Although no direct correlation to a viral gene expression factor that regulates pathogenicity has been established, results so far suggest that this antibody has potential for rapidly identifying severe isolates of CTV that cause economic injury in major citrus cultivars. In Spain and California, seedling yellows and stem pitting isolates of CTV have been introduced from other citrus-growing regions. These isolates threaten major production areas (2,4,13) unless they can be identified and removed before extensive secondary spread occurs. The CTV-MCA13 monoclonal antibody could be used in screening assays to avoid propagation of trees infected with severe isolates. For example, some of the approximately 55,000 registered budwood source trees in Florida have become infected with decline-inducing isolates of CTV. These trees remain symptomless because they are grafted on CTV-tolerant rootstocks, but when they are propagated on sour orange, they cause severe stunting. It is not practical to index large numbers of budwood trees at frequent intervals on citrus indicator plants, but this could be done readily by ELISA with a discriminating antibody such as CTV-MCA13 (7).

There also has been intense interest in mild strain cross protection as a control strategy for CTV (18,21,22). A major obstacle has been lack of a rapid means to evaluate protective ability of mild strains. Use of discriminating antibodies such as CTV-MCA13 could greatly assist evaluation of replication and movement of the challenge isolate after inoculation. It also could allow quantitative evaluation of results.

Although use of known quantities of purified antigen facilitates accurate evaluation of strain discrimination, we believe that, with suitable reference controls, crude antigen sources can be used effectively. Where the ultimate goal is a probe for practical application, crude extracts are preferable because they simulate the actual field samples that would be assayed. Freeze-dried crude antigen extracts provided a uniform source of CTV isolates. This factor was especially useful for early screening of hybridoma cell lines.

The high activity of CTV-MCA13 in PTA indirect ELISA is a favorable property for large-scale screening applications. Neither the polyclonal antisera tested nor 3DF1 show strong affinity to plate-trapped CTV antigens. Development of a more effective broad spectrum reference monoclonal antibody with high affinity for plate-trapped antigens would be desirable.

Additional testing will be required to define the range of isolate discrimination achievable with CTV-MCA13 and to characterize the epitope responsible for the specificity, but we believe that results from this study clearly demonstrate the potential for serological discrimination of CTV isolates.

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