Use of Specific Monoclonal Antibodies for Diagnosis of Citrus Tristeza Virus

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ABSTRACT. Monoclonal antibodies (MAb) specific for citrus tristeza virus (CTV) were obtained by fusion of a nonsecreting myeloma cell line with spleen cells from immunized BALB/c mice. Immunoglobulin titre and isotype were determined in cell culture supernatants and mouse ascitic fluid. Each MAb was enzyme-conjugated and used in a double-antibody sandwich ELISA assay for CTV. All reacted uniformly against a panel of 23 strains of CTV representative of strains collected from Europe, Asia, and North America.
Index words. immunoenzymatic techniques, antigenic regions.

Tristeza, the most important viral disease of citrus, is caused by citrus tristeza virus (CTV) a closterovirus that is disseminated in the field by aphids.

Usually, testing for CTV has been performed by graft inoculation on Mexican lime seedlings, but this method is expensive and results are obtained only after several months. In recent years, several serological methods have been developed for specific and rapid detection of CTV: SDS-double immunodiffusion (12), ELISA (2, 3), immunoelectron microscopy (13), immunofluorescence (28), and radioimmunoassay (22). ELISA has been particularly useful for large-scale CTV surveys (9, 23) and has introduced new possibilities for the study and control of the disease (4, 14, 24). These studies have used conventional antisera (polyclonal antibodies, PAb). Recently, by hybridoma technology (20), monoclonal antibodies (MAb) have been obtained to tobamoviruses (1, 8), potyviruses (15), potexviruses (27) luteoviruses (7, 18) and ilarviruses (16).

In this paper, we describe the preparation and characterization of CTV-specific monoclonal antibodies and their use for the diagnosis of CTV infection.

MATERIALS AND METHODS

Virus isolates. Twenty CTV isolates representative of different citrus areas in Spain were transmitted by *Aphis gossypii* (17) to healthy citrus plants to separate CTV from other non-aphid-transmissible citrus viruses. Purified isolates were graft-transmitted to uniform groups of healthy sweet orange and Mexican lime seedlings grown in a controlled-temperature greenhouse (17 to 24 C). CTV isolate T-514 from California (provided by Dr. D. J. Gumpf, Univ. California, Riverside, U.S.A.), and two unclassified isolates from Iran (provided by Dr. V. Minassian) and Vietnam (provided by Dr. N. Matos, Sanidad Vegetal, La Habana, Cuba) were also assayed.

Virus purification. Isolate T-308 was purified from calamondin according to the method previously described by Flores *et al.* (10) with slight modifications. Briefly, leaves of infected plants were homogenized in five volumes 0.05 M Tris-HCl, pH 8.0. Virus was precipitated by the addition of polyethylene glycol 6,000 to 4% and NaCl to 0.8% and incubated for 16 hr at 4 C. The precipitate was resuspended in 0.1 M phosphate buffer (PB), pH 8.2 and was centrifuged at 100,000 *g* for 90 min. This pellet was resuspended overnight at 4 C. Excess contaminating plant proteins were precipitated by the addition of ammonium sulfate to 33% saturation and incubation for 2 hr at 4 C. The supernant was exhaustively dialysed against PB, and the virus was collected by centrifugation at 100,000 *g* for 3 hr. This semipurified virus stock

was resuspended in 0.1 M PB and stored at –70 C.

Polyclonal antibodies. The following antisera specific for CTV were used: 879, 873 and 894 (provided by Dr. S. M. Garnsey, USDA, Orlando, FL, U.S.A.), R4 (provided by Dr. D. J. Gumpf) and M1 provided by (M. Cambra *et al.*).

Production and characterization of hybridomas secreting MAb specific for CTV. Production of hybrid cells secreting MAb specific for CTV was performed by fusion between a nonsecreting mouse myeloma and spleen cells from CTV-immunized mice. BALB/c mice were immunized by intraperitoneal injection of 0.1 ml CTV preparation (20 μg protein) emulsified in an equal volume of Freund's complete adjuvant (Difco). Fifteen and 30 days later, mice were injected with the same amount of virus emulsified in Freund's incomplete adjuvant. For 3 days before fusion, mice were injected daily with 10 μg CTV. Hybridization was carried out by mixing $4x10^8$ spleen cells with $1x10^8$ cells of the mouse myeloma X63/Ag8-653 (20) and fusing them using polyethylene glycol 1,000 (Koch-Light) following the procedure described by Nowinski *et al.* (25) Cells were cultured under selective conditions (HAT medium) in 96-well Costar trays. Between 10 and 17 days after fusion the cell-free culture fluids were screened for the presence of antibodies against CTV using an indirect ELISA (see below). Specific antibody-secreting hybridomas were cloned under conditions of limiting dilution with peritoneal macrophages and thymocytes as feeder layers (26). Cloning was repeated three to five times and established hybrids were grown in HT medium (HAT medium without aminopterin).

Indirect ELISA. Isotype determination, production of ascitic fluids and antibody purification were performed as described (29).

Double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-DAS). ELISA-DAS was used to test the ability of different MAb to detect CTV in crude extracts. Plant extracts were prepared with the aid of a Polytron Kinematika homogenizer using 0.2 g of young shoots of infected citrus plants in 2 ml extraction buffer (0.01 M-sodium phosphate buffer, pH 7.2, 0.14M NaCl, 0.003M NaN_3, and 1% polyvinyl-pyrrolidone 10000). The immunoassay was done in M 129A plates (Dynatech) essentially as described Clark and Adams (5) plates were coated with 2μg/ml IgG (MAb 3DF1). The standard conditions established for each type of antibody were conjugate dilutions of 1/200 (PAb 879), 1/600 (PAb R4) and 1/1000 (MAb 3DF1) and substrate incubation for 60 min. OD_{405} was measured in a Titertek Multiskan photometer (Flow Laboratories).

MAb and PAb were compared for routine CTV detection in parallel assays by ELISA-DAS using the same plant extract.

Competition antibody binding assays. Polystyrene plates (Inotech Diagnostik) were coated with 1 μg/well of a semipurified CTV preparation in 100 μl of 0.05 M carbonate buffer pH 9.6 and incubated overnight at 4 C. Plates were washed with PBS (0.15 M NaCl in 0.1M sodium phosphate pH 7.4) containing 0.02% Tween 20 (PBS-Tween 20). Twofold dilutions from 10 μg/well of unlabeled purified monoclonal antibodies and a 1:2000 dilution (500 ng) of peroxidase-labeled antibody in PBS plus 0.8% BSA and 0.05% Tween 20 was added to each well. The plate was incubated 2 hr at room temperature, washed, and substrate added. After 5 min in darkness, reaction was stopped by adding 1M H_2SO_4 and read at OD405 in a Titertek Multiskan photometer.

RESULTS

Production and characterization of monoclonal antibodies. Three fusion experiments were performed and the fusion products were plated into 800 culture wells. Growth of hybrid

cells was observed in 90% of the wells. Supernatant fluids of 98 cell cultures (12.2%) reacted positively with a CTV preparation by indirect ELISA, but only 38 of them (4.8% of the total) gave, in addition, no reaction with healthy plant extracts. Hybrid cell cultures producing the highest virus specific antibody titre were selected for cloning and further propagation. Characteristics of the seven hybrid lines established after three cloning steps are summarized in Table 1. Three of them were used in further experiments.

Detection of CTV with MAb. The MAb and several PAb were used to detect CTV by ELISA-DAS, ELISA-IDAS and IEM in crude extracts of plants infected with isolates T-300 or T-308, isolates known to differ in aphid transmissibility and intensity of symptoms induced in Mexican lime (18). CTV could be detected by ELISA-DAS and ELISA-IDAS (trapping the antigen with either MAb or PAb) in crude extracts using MAb 3DF1, 3BH6 and 3CA5.

MAb 3DF1, 3BH6 and 3CA5 reacted positively by ELISA-DAS with 20 CTV isolates from different Spanish citrus areas, as well as with CTV isolates from California (T-514) and two unclassified isolates from Iran and Vietnam.

Table 2 summarizes the results obtained with the different Spanish CTV isolates using the three MAb. The highest differences between absorbance values of extracts from healthy and CTV-infected plants were obtained with 3DF1, which was therefore used further for comparison with PAb and for routine detection of CTV. Absorbance values lower than 0.1 were obtained with healthy plant extracts from Mexican lime, sweet orange, sour orange grapefruit, Clementine mandarin or satsuma mandarin even after 19 hr of incubation with the substrate.

Comparison of MAb and PAb for the detection of different CTV isolates by ELISA-DAS. Extracts of Mexican lime plants infected with 20 CTV isolates were assayed by ELISA-DAS using PAb 879 and R4, and MAb 3DF1. The controls were a mixture of young shoots from healthy Mexican lime (negative) and a mixture of young shoots from 350 Mexican lime seedlings infected with T-308 and kept in the greenhouse for 1 yr (positive). Tissue was freeze-dried

TABLE 1
CHARACTERIZATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR CITRUS TRISTEZA VIRUS (CTV)

Hybridomas	Isotype	Supernatant titer[z]	IgG titer[y]
1. *3DF1*	IgG_{2b},K	15,525	1 ng/ml
2. *2BA11*	IgG_3,K	25	1 μg/ml
3. *3BH6*	IgG_3,K	390,625	0.01 ng/ml
4. *3CA5*	IgG_{2b},K	15,625	0.1 μg/ml
5. *4BH4*	IgG_1,K	15,625	10 ng/ml
6. *4CF3*	IgG_{2b},K	9,375	0.1 ng/ml
7. *4AH2*	IgG_1,K	625	—

[z]Supernatant titre determined by indirect immunoenzymatic assay and expressed as reciprocal of dilution endpoint.
[y]IgG titer determined by indirect immunoenzymatic assay. IgG purified from ascites and concentration adjusted to 1mg/ml.

TABLE 2
REACTIVITIES OF THREE CITRUS TRISTEZA VIRUS (CTV)-SPECIFIC MONOCLONAL ANTIBODIES AGAINST 20 DIFFERENT SPANISH CTV ISOLATES AS DETERMINED BY ELISA-DAS

Monoclonal antibody	OD_{405}		
	Positive standard	Mean of 20 Spanish isolates	Negative standard
3DF1	1.665	1.424 ± 0.210	0.013
3BH6	0.983	0.925 ± 0.084	0.001
3CA5	0.777	0.838 ± 0.074	0.020

and kept at –20 C. The results are shown in fig. 1. The absorbance values are the means of eight repetitions of two different experiments. With most CTV isolates higher absorbance values were obtained using MAb 3DF1 than with the two PAb assayed. In addition, absorbance values of the healthy plant extracts were lower with MAb 3DF1 than with PAb. Variation was observed among absorbance values obtained with each type of antibody and with different CTV isolates.

Comparison of the binding characteristics of different monoclonal antibodies: evaluation of relative avidities. Six different monoclonal antibodies CTV-specific were compared for activity in our antibody-binding assay. The avidities of the monoclonal antibodies was determined according to Frankel and Gerhard (12) (data not shown). Fig. 2 shows graphically the results typically obtained in competition binding assays. These experiments done with the six different MAb (fig. 3) allowed a preliminary definition of at least two antigenic regions on the CTV coat protein; one of them is well defined by the three MAb of higher avidity: 3BH6, 3CA5 and 3DF1.

DISCUSSION

Seven hybridomas secreting CTV-specific antibodies have been obtained. The antibodies produced by three of these gave high titre and were IgG. These MAb reacted strongly with preparations of CTV in indirect ELISA, ELISA-DAS or ELISA-IDAS. Also, MAb 3DF1, 3BH6 and 3CA5 reacted with CTV in crude or semipurified extracts of infected plants in ELISA-DAS. ELISA-IDAS or IEM, but not in double immunodiffusion test in the presence of SDS (data not shown).

The MAb reacted positively with 20 CTV isolates in ELISA-DAS, which suggests that each of the three MAb recognizes a serological determinant common to all isolates. MAb 3DF1 also reacted with CTV isolates from other countries, some of which contain the seedling yellows component. The other MAb, 3BH6 and

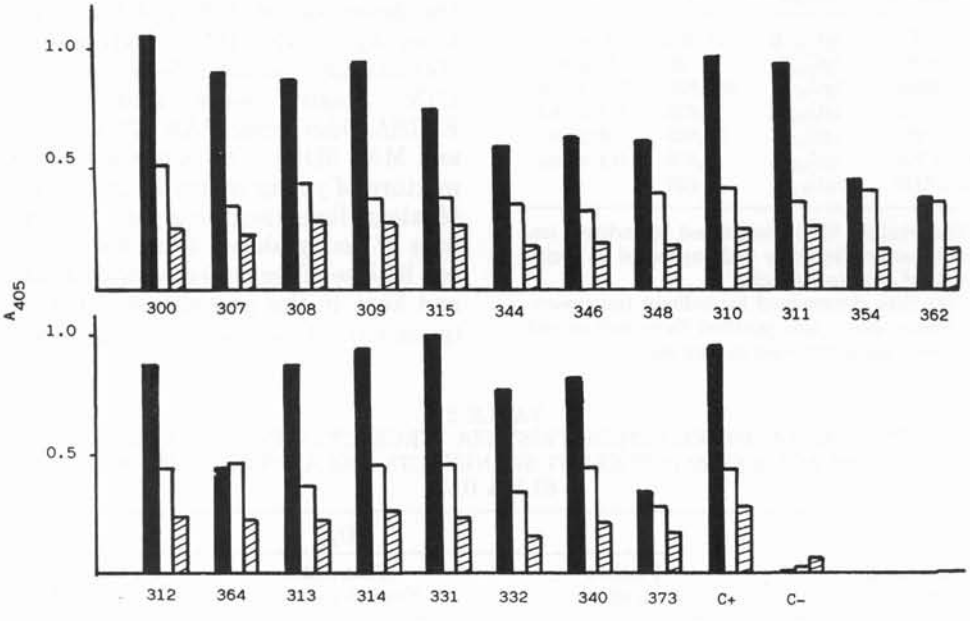

Fig. 1. ELISA-DAS results comparing monoclonal (MAb) and polyclonal (PAb) antibody reactions to different CTV isolates (300 to 373), infected Mexican lime (as positive control, C+), and healthy tissue (as negative control, C–). IgG was from MAb 3DF1 (■), PAb 879 (□) and PAb R4 (□).

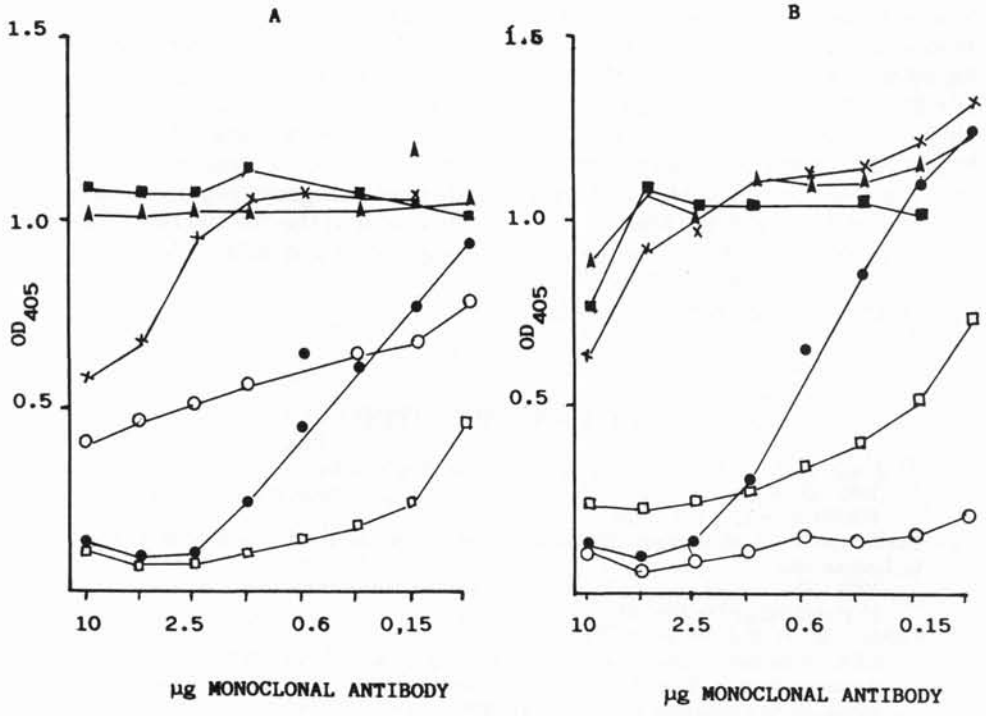

Fig. 2. Competition binding assay. Graphic shows data obtained in a competition binding assay. A) 3BH6 is the peroxidase-labeled MAb. B) 3DF1 is the labeled MAb. Competitor non-labeled MAb are defined as: (○) 3DF1, ● 3BH6, X 2BA11, □ 3CA5, ■ 4BH4 and Δ 4CF3.

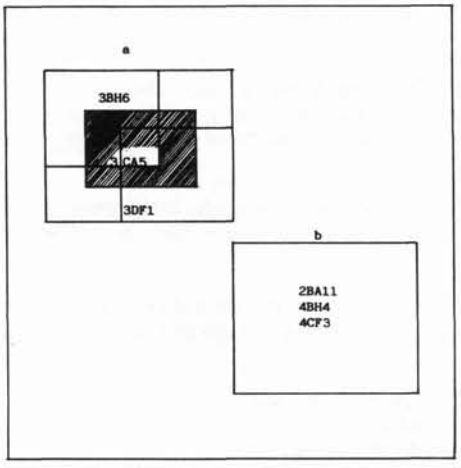

Fig. 3. A tentative antigenic map is shown based on competitive binding assays. Two different antigenic regions have been determined; one of them has at least three different epitopes defined by monoclonal antibodies 3BH6, 3DF1 and 3CA5. No data are available about the other antigenic region.

3CA5, were not tested against these exotic isolates.

Low absorbance values obtained with negative controls using MAb 3DF1 enhanced the differentiation between infected and healthy plants and made CTV identification more certain than by using PAb. The low background remains even after long incubation with the substrate (up to 24 hr). Consequently, high dilutions of MAb conjugate can be combined with longer periods of incubation to save conjugate and still obtain clean plates.

So far, results obtained in routine detection of CTV in the field indicate that any tree found infected by PAb tests will also give a positive ELISA reaction with MAb. In addition, some trees that gave a negative ELISA reaction with PAb were found to be CTV-infected using MAb 3DF1. When tested again some 3 months later with PAb and 3DF1, the same trees gave positive results with both types of antibody (data not shown).

These observations demonstrate the higher sensitivity of MAb 3DF1 compared with PAb and rule out the possibility of false positive results in the earlier assays. This difference between MAb and PAb is an indication that higer sensitivity is obtained with MAb when testing field trees.

Variation between absorbance values obtained using different isolates might perhaps reflect antigenic variation, but it is equally possible that the variation reflects only different concentrations of virus antigen in the infected tissues.

Preliminary data of an epitopic map of the CTV protein showed two distinct antigenic regions that could be useful in the "distinction" between aggressive and mild CTV strains (fig. 3).

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