

Key words: CTV/monoclonal antibodies/diagnosis

## Production and Characterization of Monoclonal Antibodies Specific for Citrus Tristeza Virus and Their Use for Diagnosis

By C. VELA,<sup>1</sup>\* M. CAMBRA,<sup>2</sup> E. CORTÉS,<sup>1</sup> P. MORENO,<sup>2</sup>  
J. G. MIGUET,<sup>1</sup> C. PÉREZ DE SAN ROMÁN<sup>1</sup> AND A. SANZ<sup>1</sup>

<sup>1</sup>*Inmunología y Genética Aplicada SA (INGENASA), Hermanos García Noblejas 41, 28037 Madrid and* <sup>2</sup>*Instituto Valenciano de Investigaciones Agrarias (IVIA), Apartado Oficial, Moncada, Valencia, Spain*

(Accepted 30 September 1985)

### SUMMARY

Monoclonal antibodies (MAB) specific for citrus tristeza virus (CTV) were obtained from hybrid cells produced by fusion of a non-secreting myeloma cell line with spleen cells from BALB/c mice immunized with isolate T-308 of CTV. Three MAB were characterized for their immunoglobulin isotype and their titres in cell culture and ascites fluids. Each MAB was conjugated with alkaline phosphatase and used in a double-antibody sandwich enzyme-linked immunosorbent assay for CTV. When tested against 23 strains of CTV, each MAB recognized all the strains with uniform reactions and low background.

### INTRODUCTION

Tristeza is the most important virus disease of citrus worldwide. It is caused by citrus tristeza virus (CTV), a closterovirus that is naturally spread in the fields by aphids.

Usually, testing for CTV has been by graft inoculation on Mexican lime seedlings [*Citrus aurantifolia* (Christm) Swing.], 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 (Garnsey *et al.*, 1978), ELISA (Bar-Joseph *et al.*, 1979; Cambra *et al.*, 1979), immunoelectron microscopy (Garnsey *et al.*, 1980), immunofluorescence (Tsuchizaki *et al.*, 1978), and radioimmunoassay (Lee *et al.*, 1981). ELISA has been particularly useful for large-scale CTV surveys (Moreno *et al.*, 1980; Fishman *et al.*, 1983) and has introduced new possibilities for the study and control of the disease (Cambra *et al.*, 1981; Garnsey *et al.*, 1981; Müller & Garnsey, 1984). These studies have used conventional antisera (polyclonal antibodies, PAb). Recently, by hybridoma technology (Köhler & Milstein, 1975) monoclonal antibodies (MAB) have been obtained to tobamoviruses (Dietzgen & Sander, 1982; Al Moudallal *et al.*, 1982), potyviruses (Gugerli & Fries, 1983), potexviruses (Sanz *et al.*, 1985*b*), luteoviruses (Diacó *et al.*, 1983; Hsu *et al.*, 1984) and ilarviruses (Halk *et al.*, 1984).

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

### METHODS

*Virus isolates.* Twenty CTV isolates representative of different citrus areas in Spain were transmitted by *Aphis gossypii* (Hermoso de Mendoza *et al.*, 1984) 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 [*Citrus sinensis* (L) Osb.] and Mexican lime seedlings grown in a controlled temperature greenhouse (18 to 25 °C). CTV isolate T-514 from California (provided by Dr D. J. Gumpf, University of California, Riverside, Ca., 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.

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

**Virus purification.** Isolate T-308 was purified from calamondin (*Citrus madurensis* Lour.) according to the method previously described by Flores *et al.* (1974) with slight modifications. Briefly, leaves of infected plants were homogenized in 5 vol. 0.05 M-Tris-HCl pH 8. Virus was precipitated by the addition of polyethylene glycol 6000 to 4% and NaCl to 0.8% and incubation for 16 h at 4 °C. The precipitate was resuspended in 0.1 M-phosphate buffer pH 8.2 (PB) and was centrifuged at 100000 g for 90 min. This pellet was resuspended overnight at 4 °C. Excess contaminating plant proteins were precipitated by the addition of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> to 33% saturation and incubation for 2 h at 4 °C. The supernatant was exhaustively dialysed against PB, and virus was collected by centrifugation at 100000 g for 3 h. This semi-purified virus stock was resuspended in 0.1 M-PB and stored at -70 °C.

**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 non-secreting 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 complete Freund's adjuvant (Difco). Fifteen and 30 days later, mice were injected with the same amount of virus emulsified in incomplete Freund's adjuvant. For 3 days before fusion, mice were injected with a daily dose of 10 µg CTV. Hybridization was carried out by mixing  $4 \times 10^8$  spleen cells with  $1 \times 10^8$  cells of the mouse myeloma X63-Ag8-653 (Kearney *et al.*, 1979) and fusing them using polyethylene glycol 1000 (Koch-Light) following the procedure described by Nowinski *et al.* (1979). 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 (Sanz *et al.*, 1985a). Cloning was repeated three to five times and established hybrids were grown in HT medium (HAT medium without aminopterin).

**Indirect ELISA.** This was used to detect specific antibodies for CTV in supernatant fluids of the cell cultures. Polystyrene plates (Inotech Diagnostik) were coated with 1 µg/well of a semi-purified preparation of the homologous CTV strain in 100 µl carbonate buffer (0.05 M, pH 9.6), and incubated overnight at 4 °C. Plates were washed with PBS (0.15 M-NaCl in 0.1 M-sodium phosphate pH 7.4) containing 0.02% Tween 20 (PBS-Tween) and 100 µl/well of undiluted cell-free culture fluid was pipetted into the wells. Peroxidase-labelled rabbit anti-mouse IgG (heavy and light chains) was used as the conjugate. As a control, plates were coated with a filtered extract of healthy calamondin plants made in carbonate buffer (1:10, w/v).

Reactions were considered positive when the absorbance readings were three or more times greater than those obtained when the supernatant fluid from secreting myeloma P3-X63-Ag8 (Köhler & Milstein, 1975) was used in place of the MAb.

**Determination of MAb isotype.** MAb isotypes were determined by double immunodiffusion with antisera specific for mouse heavy and light immunoglobulin chains (Litton Bionetics, Kensington, Md., U.S.A.). MAb from cell culture fluids were precipitated with ammonium sulphate and concentrated tenfold. Immunodiffusion tests were made in 1% Noble agar (Difco) in veronal buffer at pH 7.4 (Ouchterlony & Nilsson, 1978). Precipitation lines were observed after 24 to 48 h.

**Production of ascitic fluids.** Ascitic fluid was produced by intraperitoneal inoculation of  $2 \times 10^6$  to  $8 \times 10^6$  hybrid cells into syngeneic BALB/c mice that had been injected 3 to 20 days before with 0.5 ml Pristane (2,6,10,14-tetramethylpentadecane, Aldrich).

**Antibody purification.** MAb were purified from ascitic fluid by affinity chromatography on Protein A-Sepharose (Kronvall *et al.*, 1970). Purity of the immunoglobulin was checked by SDS-polyacrylamide gel electrophoresis.

**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, 0.14 M-NaCl, 0.003 M-NaN<sub>3</sub>, 1% polyvinylpyrrolidone 10000). The immunoassay was done in M129A plates (Dynatech) essentially as described by Clark & Adams (1977). A<sub>405</sub> 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.

**Indirect double-antibody sandwich enzyme-linked immunosorbent assay (ELISA-IDAS).** ELISA-IDAS was done as described by Bar-Joseph & Malkinson (1980). Briefly, plates were coated with MAb (1 µg/ml) or PAb (2 µg/ml) and plant extracts prepared as described were added. When plates were coated with MAb the assay was completed by adding PAb (5 µg/ml) and goat anti-rabbit IgG (Behring Institute) conjugated with alkaline phosphatase as described above. In plates coated with PAb the assay was completed by adding MAb (2 µg/ml) and peroxidase-conjugated rabbit anti-mouse IgG (Cappel Laboratories).

**Immunoelectron microscopy (IEM).** Grids were prepared as described by Derrick & Brlansky (1976) using Parlodion or Formvar films and immunoglobulins (50 µg/ml) from different MAb and PAb (873, 879, R4 and M1).

Table 1. Characteristics of hybrid lines secreting CTV-specific antibodies

Hybrid line	Antibody isotype	Titre by indirect ELISA*	
		Culture fluid	IgG†
3DF1	IgG2b K	15625	10 <sup>6</sup>
3BH6	IgG3 K	390625	10 <sup>4</sup>
3CA5	IgG2b K	15625	10 <sup>4</sup>

\* Reciprocal of dilution endpoint.

† IgG purified from ascites and concentration adjusted to 1 mg/ml.

Table 2. Reactivities of three CTV-specific monoclonal antibodies against 20 different Spanish isolates, as determined by ELISA-DAS

Monoclonal antibody	$A_{405}$		
	With positive standard	Mean with 20 Spanish isolates	With 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

An antiserum to *Agrobacterium radiobacter* pv. *tumefaciens* was used as a negative control. Plant extracts were prepared as described by Garnsey *et al.* (1980) using young shoots of Mexican lime infected with CTV isolates T-300 and T-308. Observations were made in Jeol JEM-100 S electron microscope.

## 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 them. 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 extract. Hybrid cell cultures producing the highest antibody titre were selected for cloning and further propagation. Characteristics of the three hybrid lines established after three cloning steps are summarized in Table 1.

### *Identification 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 (Hermoso de Mendoza *et al.*, 1984). 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.

Detection of CTV isolates T-300 by IEM was accomplished with MAb 3DF1, 3BH6 and 3CA5. Detection of the two CTV isolates was achieved with the four PAb assayed, even though none of these antisera was raised against isolates T-300 or T-308. No difference was observed between grids prepared with Formvar or Parlodion and coated with PAb or with MAb. Grids prepared with T-300 isolates showed five to ten particles per field at ×21000 magnification, whereas those prepared with T-308 had 20 to 50 particles per field. In grids coated with control antiserum only one or two particles could be found every five to ten fields at the same magnification.

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 from Iran and Vietnam.

Table 2 summarizes the results obtained with the different Spanish CTV isolates using the three MAb. The highest differences between *A* values of extracts from healthy and CTV-

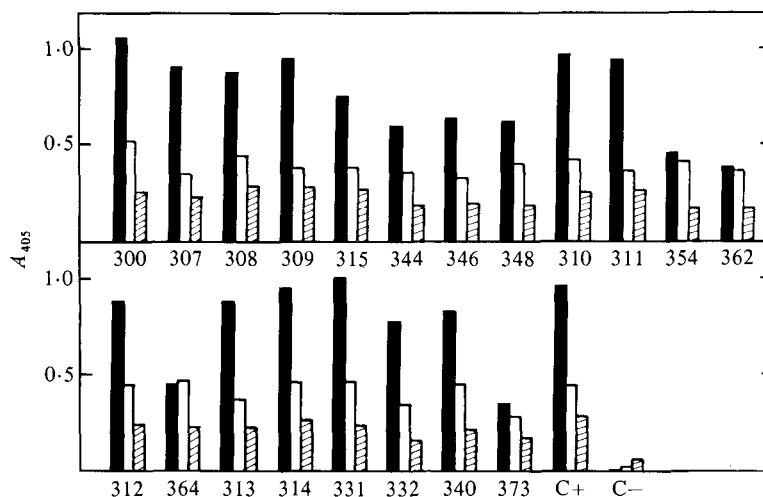


Fig. 1. Histogram comparing mono- and polyclonal antibodies in ELISA-DAS with isolates of CTV (300 to 373), infected Mexican lime (positive control, C+) or healthy tissue (negative control, C-). IgG was from MAb 3DF1 (■), PAb 879 (□) or PAb R4 (▨).

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 (*C. aurantium* L.), grapefruit (*C. paradisi* Macf.), clementine mandarin (*C. clementina* Host. ex Tan) or satsuma mandarin [*C. unshin* (Mark.) Mare] even after 19 h 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 year (positive) (shoots close-trimmed, freeze-dried and kept at  $-20^{\circ}\text{C}$ ). The results are shown in Fig. 1. The  $A$  values are the means of eight repetitions of two different experiments. Plates were coated with  $2\ \mu\text{g/ml}$  IgG (PAb 879 and PAb R4) and  $1\ \mu\text{g/ml}$  IgG (MAb 3DF1). Conjugate dilutions were 1/200 (PAb 879), 1/600 (PAb R4) and 1/1000 (MAb 3DF1); substrate was incubated for 60 min. These are the standard conditions established for CTV detection with each type of antibody. With most CTV isolates higher  $A$  values were obtained using MAb 3DF1 than with the two PAb assayed. In addition,  $A$  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.

#### *Routine detection of CTV with MAb and PAb by ELISA-DAS*

Extracts from 1795 samples of field trees were simultaneously tested by ELISA-DAS using MAb 3DF1 and PAb 879 or R4 under standard laboratory conditions (see legend of Fig. 1) for routine indexing. Positive reactions with both MAb and PAb were obtained for 584 samples, 1199 were found negative in both assays and 12 were found negative with PAb but positive with MAb 3DF1.

#### DISCUSSION

Three hybridomas secreting CTV-specific antibodies have been obtained. The antibodies produced by each of three high titre clones established 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 semi-purified extracts of infected plants in ELISA-DAS, ELISA-IDAS or IEM, but not in double immunodiffusion tests 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. Other MAbs, 3BH6 and 3CA5, were not tested against these exotic isolates.

Low absorbance values obtained with negative controls using MAb 3DF1 enhance the differentiation between infected and healthy plants and make CTV identification easier than it is when using PAb. The low background remains even after long periods of incubation with the substrate (up to 24 h at 4 °C). Consequently, high dilutions of MAb conjugate can be combined with longer periods of incubation to obtain clean plates, thereby saving conjugate.

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 particles in the infected tissues.

Results obtained so far 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 higher sensitivity is obtained with MAb when testing field trees.

The authors would like to thank Miss A. Sebastian for secretarial assistance in the preparation of the manuscript, and Dr J. R. Allen for critical discussion of it.

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(Received 23 April 1985)