Development and Characterization of Monoclonal Antibodies to Citrus Tristeza Virus (CTV) Strains in Taiwan

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ABSTRACT. Twelve stable hybridoma cell lines secreting CTV-specific antibodis were obtained against purified sources of SY-M, a mild seedling-yellows strain of CTV, and CTV-D, a non-seedling-yellows dwarfing strain. These monoclonal antibodies (MAB) included IgG 1, IgG 2a, and IgM subtypes. Immunoglobulin titers up to 4,096 X and 83,886,080 X were obtained in culture supernatants and ascites fluid respectively. To MABs were found which reacted differently to mild and severe CIV strains in double antibody sandwich-indirect ELISA. Thirteen domestic and foreign isolates of CTV showed six distinct reaction patterns when tested with the two MABs from Taiwan plus MAB MCA13 from Florida and MAB 3DF1 from Spain The MAB 4H6-1 is distinct from previously described MAB. The MAB 10E3-4 reacts similarly to MCA13, but shows at least some quantitative differences with several isolates.
Index words. ELISA, stem pitting, serotypes, pummelo

Citrus tristeza virus (CTV) is endemic in citrus in Taiwan. Strains of CTV vary in severity from very mild ones which cause little or no visible damage to those which cause severe symptoms. Since all citrus cultivars in Taiwan are propagated on CTV-tolerant rootstocks, the primary economic problem is that certain CTV isolates cause stem pitting in pummelo (8). Major efforts are underway to rehabilitate the citrus industry in Taiwan which has been severely damaged by likubin (greening). Extensive new plantings of virus-free trees are being made in many locations on the island. Reinfection of these trees by CTV is expected, but it is uncertain what the severity of the prevalent reinfecting strains will be. Development of protective mild strains is desired, and a means to rapidly evaluate protective ability of candidate mild isolates is needed.

A program was initiated several years ago in Taiwan to develop monoclonal antibodies (MABs) to CTV which could differentiate mild and severe isolates of CTV. Production of MABs to CTV has been previously reported (5). MABs generated previously to CTV in Spain reacted to all isolates originally tested regardless of severity (9). Subsequently, however, the MAB CTVMCA13 produced in Florida differentiated mild and severe isolates of CTV (7). Recently, comparative tests of several different Florida and Spanish MABs indicate that a number of different epitopes exist in the CTV coat protein and that none of the MABs described to date react to all isolates (3).

In this paper, we describe production of MABs to two Taiwanese isolates of CTV and the reactivity of MABs produced to differential isolates of CTV. Evidence is presented that at least one of the Taiwan MABs is specific to a previously undescribed epitope of the CTV coat protein.

MATERIALS AND METHODS

Virus isolates. Pure Taiwanese CTV isolates were obtained through bioassay or aphid transmission of field sources pf CTV. These CTV isolates were differentiated biologically into different strains according to symptom expression in Eureka lemon, sour orange and grapefruit seedlings, Mexican lime grafted on rough lemon, and Wentan pummelo or Peiyu pummelo. Other isolates of CTV were kindly provided by Dr. S. M. Garnsey as freeze-dried tissue extracts from the USDA Beltsville Agricultural Research Center collection (2).

Production and characterization of MABs specific for CTV. A mild seedling-yellows strain (SY-M) and a non-yellows source which causes severe

stem pitting and dwarfing in pummelo (CTV-D) (8) were used as sources of immunogen. The SY-M source was coded as Tm-1 and the CTVD source was coded as PYdw-CY-5-2. The isolates were propagated separately in rough lemon and purified as described by Vela *et al.* (9). Purified virus was used to immunize BALB/C mice. Three intraperitoneal injections were made at 4-week intervals. Each injection was made with an emulsion of 0.1 ml of CTV preparation (50 μg protein) and an equal volume of Freund's complete adjuvant (Difco) (except the last injection). The mice were sacrificed three days after the last injection. Fusion procedures used were essentially those previously described (4, 6). Spleen cells were harvested and added to myeloma cells (NS-1) at a 5-to-1 ratio (6). Cell fusion was done by adding 1 ml of 45% polyethylene glycol (MW 1,500 Merck) to the pelleted mixture of cells. The mixture was incubated in a 37C water bath for 75 sec, slowly diluted with 2 ml RPMI-1640 (6), and incubated at 37C for 60 sec. The cycle was repeated once and 20 ml of RPMI-1640 was slowly added (120 sec). The fused cells were centrifuged at 1,000 g for 10 min. The pellet was resuspended in HAT-selected medium and cultured in 96-well microplates (Nunc). Between 10 and 20 days after fusion, culture supernatants of viable cells were screened individually for production of antibodies specific against CTV by indirect ELISA (1). Hybridoma cells secreting CTV-specific antibodies were cloned twice by limiting dilution and grown with thymocytes as feeder cells in HT medium or in RPMI-1640 with 15% fetal bovine serum. MABs isotypes were determined by using a MAB-isotyping kit (PharMingen) (4). Additional information on techniques is described by Harlow and Lane (6).

ELISA. The indirect ELISA method (1) was used for screening CTV-specific antibody secreted by hybridomas. Virus antigens in crude plant extract (1/5 dilution W/V), were trapped on ELISA microtiter plates (Nunc) during overnight incubation at 4C. The coated microplates were blocked with 0.1% BSA for 1 hr (5), washed, loaded with culture supernatant, and incubated at 37C for 2 hr. Anti-mouse immunoglobulin labeled with alkaline phosphatase (KPL) was added to the microplates and incubated at 37C for 2 hr. Finally, substrate solution (1 mg/ml) was added and plates were incubated at 37C for 1 hr. The results were read by an ELISA plate reader to determine the OD_{405} of the solution.

The Double Antibody Sandwich Indirect (DASI) method was used for epitope diversity analysis of MABs as described previously by Permar *et al.* (7).

Other antibodies sources. The epitope diversity of the Taiwanese MABs obtained was compared with MCA13 provided by S. M. Garnsey and T. A. Pamar, and MAB 3DF1 provided by M. Cambra. The polyclonal antibody used was from 1052 antisera to the Florida CTV isolate T36 (7) provided by S. M. Garnsey.

RESULTS

Twelve stable hybridoma cell lines secreting CTV-specific MAB were obtained. Three were to CTV-D isolate PYdw-CY-5-2 and nine were to SY-M isolate Tm-1. Their antigenic specificity, as shown by ELISA, is summarized in Table 1. The immunoglobulin tissues of the MABs obtained were identified at IgG1, IgG2a, and IgM subtypes Table 1).

Two MABs, coded as 4H6-1 and 10E3-4, were selected for further testing of antigenic specificity with selected CTV isolates collected from Taiwan. Three distinct seroreaction patterns were found (Table 2). All of the severe seedling yellows strain (SY-S) isolates reacted strongly to moderately with both MABs. The pummelo dwarfing isolates of CTV (CTV-D) also showed strong seroreaction with both MABs. Mild isolates of seedling yellows strain (SY-M) reacted with 4H6-1, but either did not react or reacted weakly with 10E3-4. One mild isolate of CTV (CTV-M) showed a strong reac-

TABLE 1
ELISA REACTIONS OF SELECTED TWICE-CLONED MONOCLONAL HYBRIDOMA CELL LINES TO EXTRACTS OF HEALTHY AND CITRUS TRISTEZA VIRUS-INFECTED CITRUS

CTV strain[z]	Primary hybridoma line	Hybridoma subclone (series no.)		Isotype	OD_{405}	
					CTV	Healthy[y]
SY-M[z]	3D10	3D10F9E7	(3D10-1)	IgG2a[x]		
	4H6	4H6H12F12	(4H6-1)	IgG-1	1.412	0.254[w]
		4H6H12G2	(4H6-2)	IgG-1	1.085	0.098
	10B1	10B1E5	(10BE3-1)	IgG-1	2.000	0.424
	10E3	10E3A10A5	(10E3-1)	IgG-1	1.094	0.015
		10E3C3B4	(10E3-2)	IgG-1	1.028	0.018
		10E3E4B11	(10E3-3)	IgG-1	1.584	0.009
		10E3H2B9	(10E3-4)	IgG-1	1.032	0.010
CTV-D[z]	3G6	3G6C1A9	(3G6-1)	IgG-M	0.913	0.179
		3G6C10D9	(3G6-2)	IgG-M	1.394	0.314
		3G6C10D11	(3G6-3)	IgG-M	0.824	0.279

[z]CTV isolates used for immunizing BALB/C mice were SY-M isolate Tm-1 and CTV-D isolates PYdw-CY-5-2.
[y]Healthy = crude extract of healthy citrus; CTV = extract of tissue infected with an SY-M strain or a CTV-D strain of CTV.
[x]Isotypes determined by MAB isotyping kit (4).
[w]ELISA values read 1 hr after incubation at 37C.

tion with 4H6-1 and no reaction with 10E3-4, while another CTV-M isolate (Th-HC-89-5) which reacted weakly with polyclonal antibody did not react with either MAB. All of the CTV isolates, except one, reacted strongly with polyclonal antibodies.

The Taiwanese MABs 4H6-1 and 10E3-4 were compared with the MAB 3DF1 from Spain and the MAB MCA13 from Florida in Tests against a panel of different CTV isolates. The results (Table 3) indicate that each MAB may recognize different epitopes. MABs and 4H6-1 reacted with a wide range of isolates, but 3DF1 did not react to a Korean SY-M strain Sat/JC-S) while 4H6-1 reacted strongly to it. MAB 10E3-4 and MCS13 showed a similar seroreaction pattern. Both reacted to all SY-S isolates and did not react to CTV-M isolates and to some SY-M iso-

TABLE 2
ANTIGENIC SPECIFICITY OF MONOCLONAL ANTIBODIES (MABS) 4H6-1 and 10E3-4 PREPARED AGAINST TAIWANESE ISOLATES OF CITRUS TRISTEZA VIRUS (CTV) IN DOUBLE ANTIBODY SANDWICH INDIRECT ELISA ASSAY

CTV isolate no.	CTV strain[z]	Monoclonal antibodies		Polyclona antiserum
		4H6-1	10E3-4	
Th-IL-6-8	SY-S	++++[y]	+++	+++++
LC-86-1-1	SY-S	++++	++	+++++
WN-TT-2	SY-S	++	++	+++++
Tm-1	SY-M	++++	+	+++++
Th-YM-4	SY-M	++	−	+++
GFh-CY-5-9	CTV-M	++++	−	+++++
Th-HC-89-5	CTV-M	−	−	+
GF-CY-2	CTV-D	+++++	++++	+++++
HYdw-CY-1	CTV-D	+++++	++++	+++++

[z]SY-S = Severe seedling yellows (SY); SY-M = mild SY; CTV-M = mild, non-SY; and CTV-D = non-SY source which causes stem pitting and dwarfing in pummelo.
[y]ELISA values (OD_{405}) scored as follows: − = <0.100 and > 2X value for healthy extracts; + = 0.101 to 0.300; ++ = 0.301 to 0.700; +++ = 0.701 to 1.000; ++++ = 1.0001 to 1.500; and +++++ = > 1.501.

TABLE 3
COMPARISON OF ANTIGENIC SPECIFICITY OF TAIWANESE MONOCLONAL ANTIBODIES (MAB) 4-H6-1 AND 10E3-4 WITH MAB 3DF1 ANTIBODIES (MAB) 4H6-1 and 10E3-4 WITH MAB 3DF1 FROM SPAIN AND MCA13 FROM FLORIDA AGAINST SELECTED CTV ISOLATES FROM TAIWAN, FLORIDA AND KOREA

CTV isolate[z] no.	CTV strain[y]	Monoclonal antibodies[x]				Polyclonal Antibody
		3DR1	MCA13	4H6-1	10E4-5	
Th-IL-6-8	SY-S	++[w]	+++++	++++	++	+++++
T-36	SY-M	++	+++++	++++	+++	++++
WN-TT-57	SY-M	++	++++	++++	++	+++++
Tm-1	SY-M	+	++++	++++	+	+++++
Tm-4	SY-M	+++	+++	++	−	++++
Th-IL-11-5	SY-M	−	−	−	−	++++
Sat/JCdw-5	SY-M	−	−	++++	−	+++++
T-30	CTV-M	+++	−	++++	−	++++
GFh-CY-5-9	CTV-M	+++	−	++++	−	++++
Tm-3	CTV-M	+++	−	++	−	++
Val-86-2-3	CTV-M	++	+++	++++	++	+++++
PYdw-CY-5-2	CTV-D	+++	+++++	+++++	++++	+++++
HYdW-CY-1	CTV-D	+++	+++++	+++++	++++	+++++

[z]T-36 to T-30 are CTV isolates from Florida, Sat/Scdw-5 is from Korea, and the remaining sources are from Taiwan.
[y]SY-S = Severe seedling yellows (SY); SY-M % mild, non-SY source, CTV-D = non-SY source which causes pitting and dwarfing in pummelo.
[x]MABs 4H6-1 and 10E3-4 were prepared against Taiwan SY-M strain (Tm-1); MCA13 against the T-36 CTV isolate from Florida (8), and 3DF1 against the Spanish CTV isolate T308 (9).
[w]ELISA values (OD_{405}) scored as: − = < 0.100; + = 0.101 to 0.300; ++ = 0.301 to 0.500; +++ = 0.501 to 0.700; +++ = 0.701 to 1.000; ++++ = 1.001 to 1.500; and ++++ = >1.501.

lates. However, MCA13 reacted to SY-M isolates Tm-1 and Tm-4 while 10E3-4 reacted weakly or negatively to these same sources.

DISCUSSION

The results presented indicate that the Taiwan MABs 4H6-1 and 10E3-4 are both specific to CTV, and that each is specific to a different epitope on the coat protein. It appears that 4H6-1 is also distinct from either 3DF1 or MCA13. The MAB 10E3-4 is distinct from 3DF1, but similar to MCA13. Discrimination of some CTV isolates of SY-S, SY-M, and CTV-M strains of CTV in Taiwan can be dome serologically by panel assays with MABs against 4H6-1 and 10E3-4. It was not possible to distinguish CTV-D which causes such extensive stem pitting in pummelo trees from SY-S isolates which do not cause stem pitting in pummelo.

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