Production of Monoclonal Antibodies to Citrus Psorosis Virus

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ABSTRACT. The putative causal agent of psorosis is citrus psorosis virus (CPsV), a filamentous virus assigned to the novel genus *Ophiovirus*. Molecular (RT-PCR) and serological (ELISA) methods have recently been developed for its detection. Differences in the biological behavior of CPsV isolates, suggest that several strains of this virus may exist. In such a case, a possible way for the differential serological identification of virus strains would be the use of monoclonal antibodies (Mabs). A study for the production of CPsV-specific Mabs was therefore initiated. Mice balb/c were immunized with preparations of an Italian CPsV isolate (IAM-191Xa) purified from *Chenopodium amaranticolor*. After splenocyte fusion with myeloma cells, stable hybridoma lines secreting 24 virus-specific Mabs were selected and multiplied. These Mabs were tested singly by ELISA against a panel of 40 psorosis sources from different geographical areas (Italy, Lebanon, Spain, and USA). Sixteen different epitopes were identified and a remarkable serological variability, apparently associated with the geographical origin of the isolates, was found. None of the Mabs was able to recognize all CPsV sources, however this result was greatly affected by the virus titer.

Psorosis is an economically important and widespread disease of citrus whose putative causal agent is citrus psorosis virus (CPsV), a thread-like virus assigned to the novel genus *Ophiovirus* (8). In the recent past, the only effective method for disease identification was biological indexing on differential indicators. However, molecular (RT-PCR) and serological (ELISA) methods have recently been developed (5, 7). ELISA seems well suited for CPsV detection, but extant reagents are polyclonal antisera (7), which are not readily raised and are available in limited amounts. On the other hand, differences in the biological behavior of CPsV isolates suggest that several strains of this virus may exist. Thus, the production of monoclonal antibodies may help both in overcoming the problem of availability of serological reagents and in investigating the antigenic variability in virus populations.

García et al. (7) observed that heterologous isolates were less reliably detected than the homologous CPsV4-antigen, with both PCR and ELISA. Barthe et al. (2) and Derrick et al. (6) reported the production of monoclonal antibodies (Mabs) against strain CPV4, obtaining positive reaction only with the homologous antigen. These Mabs, as specified by Barthe et al. (2), are of little value for the diagnosis of psorosis, but they are useful for the differential serological identification of CPsV strains.

CPsV is widely spread in the citrus groves of Apulia, southern Italy (5) but it is not known whether it occurs as a single prevailing strain or as a population of multiple strains. In the hope to cast light on the extant situation and to develop a reliable protocol for the serological detection of CPsV, virus-specific monoclonal antibodies were produced and characterized as discussed in this paper.

MATERIALS AND METHODS

Virus isolate and purification. A local isolate of CPsV, named IAM-191Xa, was used as antigen source for immunization. The virus was recovered by sap transmission from a Naveline sweet orange from Apulia, and maintained and multiplied in *Chenopodium amaranticolor*, which it infects systemically (5). This makes this isolate well suited for virus purification, because of the possibility of higher virus

yields as compared with other CPsV isolates which do not infect *Chenopodium* systemically. Batches of 100-200 g of infected *C. amaranticolor* tissues were used for purification as described by García et al. (7).

Immunization and hybridoma production. Six-week old BALB/c mice (Harlan-Nossan, Correzzana) were immunized by injecting each mouse three times, at two-week intervals, with 500 μl of a CPsV IAM-191Xa preparation containing both top and bottom components, fractionated by Cs_2SO_4 gradient centrifugation, in Freunds incomplete adjuvant. A final boost, without adjuvant, was given 4 mo later, 4 d before fusion. Fusion was performed as previously described (3) with immunized splenocytes and NS0/1 myeloma cells. Hybridoma secreting CPsV-specific antibodies were first identified by analysis in DASI-ELISA of their corresponding culture supernatants on crude extracts of IAM-191Xa-infected *C. amaranticolor*, then cloned by the limiting dilution method, used for mass antibody production, both *in vivo* and *in vitro*, and frozen in liquid nitrogen.

Polyclonal antiserum. Since the selection of Mabs was by DASI-ELISA, a rabbit polyclonal antiserum, denoted as A-322, kindly supplied by R. G. Milne and E. Luisoni (Istituto di Fitovirologia Applicata, C.N.R., Turin), was used for plate coating. This antiserum had been raised using virus isolate CPsV-4, the proposed type strain of CPsV (2).

ELISA. DAS-ELISA using the antiserum A-322 was as described by García et al. (7). DASI-ELISA was used for selection and use of Mabs, as described by Cambra et al. (4).

Virus sources. A total of 64 CPsV sources were used in this study, 62 of which came from the collection of the Istituto Agronomico Mediterraneo, Valenzano, Italy. These were identified as CPsV sources because they were positive by both ELISA and graft-transmission assays (5). These sources were maintained under screen-house conditions in potted sweet orange (n = 44), clementine (n = 11), mandarin (n = 4), Satsuma (n = 2) and lemon (n = 1) trees, all grafted on sour orange. The origin of the sources was Italy (n = 38), Lebanon (n = 9), Spain (n = 2), and USA (n = 13). In particular, Italian sources were all from Apulia, the American CPsV-4 was donated by K. S. Derrick, University of Florida, whereas other American sources were donated by C. N. Roistacher, University of California. Two additional isolates (IAM-191Xa and CPsV-4q) were maintained in *C. amaranticolor* and *C. quinoa*, respectively, after mechanical transmission from their corresponding woody sources (IAM-191X and CPsV4).

Serological characterization of CPsV sources. Leaves from each virus source were homogenized in 10 vol of ELISA extraction buffer (5) and comparatively tested against each of the 24 Mabs selected (DASI-ELISA) and against antiserum A-322 (DAS-ELISA). Tests were repeated at least five times, from December 1997 to June 1998. The threshold value was arbitrarily established as three times the reading of the healthy controls, with values of no less of 0.150 OD_{405}. Virus sources were taken in consideration for their serological characterization only if at least in one of the five assays the titer was high enough in DAS-ELISA (i.e. readings over 0.300 OD_{405}, and at least six times higher than healthy controls).

As specified in the Results, 24 of 34 hybridoma lines originally obtained were retained after stabilization. The missing numbers in the progressive numbering reported in Table 2 are relative to discarded lines. Each putative epitope (that gave rise to a specific Mab) was given a letter mimicking the amino acid code, so as to construct a "string" of

16 letters for each virus source tested against the whole of the 24 Mabs. All the "strings" were comparatively analyzed using the PILEUP program (1) and the graphical expression of the alignment shown as a clustering dendrogram. Since the lack in other isolates of epitopes identified in IAM-191Xa is associated to differential serological properties, distances between isolates in the dendrogram, similarly to the analysis of amino acid sequences, can be considered as directly related to serological distances.

RESULTS AND DISCUSSION

Production of monoclonal antibodies. After fusion of immunized splenocyte with NS0/1 myeloma cells, 34 hybridoma lines secreting virus-specific Mabs were selected by DASI-ELISA. After their cloning and subcloning, repeated twice, only 24 lines proved stable and were multiplied. The corresponding Mabs, produced in vitro and in vivo (ascitic fluid), were denoted Mab.PS, each followed by one of the numbers listed in Table 1. For the serological characterization of CPsV, Mabs were used as cell culture supernatants diluted 1:10 in PBS.

ELISA and serological characterization. To identify leaves with optimal virus concentration, five young symptomatic not fully developed, and five fully expanded middle to basal leaves were compared in DAS-ELISA. In all cases, fully developed leaves gave the best results (OD_{405} values 15% to 100% higher than readings of young leaves), and therefore, fully developed leaves were used throughout.

When isolate IAM-191Xa and IAM-191X were tested in DAS-ELISA against the antiserum A-322, positive reactions were obtained 8 times out of 11 with extracts from *C. amaranticolor* and 6 times out of 11 with extracts from sweet orange (Table 1). These results were slightly better when the same two sources were tested (264 tests in total) against the whole panel of Mabs (Table 1). The outcome of these tests taken as a whole indicates that there is a high risk of false negative responses with both the polyclonal antiserum and Mabs, especially with extracts from citrus.

According to the criteria specified in "Materials and Methods" for the serological characterization of virus sources, only 40 sources of the 64 originally tested, were selected for further studies for the identification of CPsV epitopes and serogroups.

Identification of different epitopes and serogroups. The reaction of each of the Mabs obtained with each of the 40 virus sources selected are reported in Table 2. From this Table it appears that some Mabs can be grouped for their capacity to identify the same antigens. i.e.: Mab.PS1, 7, 26 and 27 (epitope A); Mab.PS13 and 21 (epitope H); Mab.PS4 and 22 (epitope C); Mab.PS24, 31, 32 and 34 (epitope P). Admitting that each of these groups represents a single epitope, and that other Mabs recognize distinct single epitopes, it can be concluded that the 24 Mabs selected identify 16 different epitopes in CPsV isolate IAM-191Xa.

As shown in Table 2, the 40 virus sources gave 14 different reaction patterns, indicating considerable serological variability, that confirms recent findings by García et al. (7) and Barthe et. al. (2). Four of these clusters were made up of two, five, seven or 16 virus sources, while the remaining ten clusters consisted of a single isolate. In the light of the above considerations on the reliability of ELISA reactions, these results may not be conclusive and should be taken with caution. The graphic representation of the clustering of different serogroups as obtained by the PILEUP program is shown in Fig. 1.

TABLE 1
POSITIVE REACTIONS OBTAINED IN 11 ELISA WITH ANTISERUM A-322 (DAS ELISA) AND EACH OF THE 24 MABS (DASI ELISA) ON THE ISOLATE IAM-191XA AND ITS CITRUS SOURCE

	IAM-191X Sweet orange	IAM-191Xa *C. amaranticolor*
Monoclonal antibody		
Mab.PS1	7/11	9/11
Mab.PS4	5/11	10/11
Mab.PS6	6/11	8/11
Mab.PS7	8/11	8/11
Mab.PS8	6/11	8/11
Mab.PS11	6/11	9/11
Mab.PS12	9/11	9/11
Mab.PS13	8/11	10/11
Mab.PS14	8/11	9/11
Mab.PS15	5/11	5/11
Mab.PS17	5/11	10/11
Mab.PS18	4/11	10/11
Mab.PS21	9/11	10/11
Mab.PS22	6/11	9/11
Mab.PS23	6/11	10/11
Mab.PS24	3/11	10/11
Mab.PS25	8/11	10/11
Mab.PS26	6/11	10/11
Mab.PS27	6/11	10/11
Mab.PS28	6/11	7/11
Mab.PS29	6/11	10/11
Mab.PS31	6/11	9/11
Mab.PS32	9/11	9/11
Mab.PS34	6/11	10/11
TOTAL DASI ELISA	154/264 (58.3%)	219/264 (82.9%)
Polyclonal antibody		
Pab A-322	6/11	8/11
TOTAL DAS ELISA	6/11 (54.5%)	8/11 (72.7%)

Correlation between geographical distribution and serological clustering of CPsV isolates. From the distribution of the subclusters in the dendrogram (Fig. 1) the following conclusions can be drawn:

(I) The 16 sources each containing all 16 epitopes (homologous-type) are all of Italian origin. These, together with source IAM-5V, which differs from the others as it lacks epitope "I", constituted a subcluster identified as the "Italian cluster".

(II) Another subcluster, containing five identical and two (P208 and P201) similar sources, all of American origin, was identified as the "American cluster".

(III) A third homogeneous subcluster, characterized by a remarkable divergence from the homologous antigen, as it lacks at least six epitopes (Table 2), contained all seven Lebanese and two American (P200 and P203) sources. This was identified as the "Lebanese cluster".

(IV) The proposed type strain CPsV-4q and its corresponding citrus source CPsV-4 did not fall into any of the subclusters. They lack four epitopes (K, Q, R, and S) but contain epitope "I", which is absent in all the other Ameri-

TABLE 2
ANALYSIS BY DASI-ELISA OF 40 CPSV SOURCES WITH 24 MABS SPECIFIC TO ISOLATE IAM191XA[z]

	Mabs.PS (and corresponding epitopes)																							
	1 1	4 C	6 D	7 A	8 E	11 F	12 G	13 H	14 I	15 K	17 L	18 M	21 H	22 C	23 N	24 P	25 Q	26 A	27 A	28 R	29 S	31 P	32 P	34 P
ITALIAN SOURCES																								
IAM-191X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-191Xa	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-190X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-194X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-195X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-197X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-318X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-365X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-374X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-393X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-9X	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-3V	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-10V	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-36V	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-39V	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-CG	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-5V	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-7V	■	■	□	■	□	■	□	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■
IAM-320X	■	■	□	■	□	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
SPANISH SOURCE																								
Sp2	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	□	■	■	■	■
AMERICAN SOURCES																								
CPV4	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	□	■	■	□	□	■	■	■
CPV4q	■	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	□	■	■	□	□	■	■	■
P203m	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■	■
P216m	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P216	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P205	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P213	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P209	■	■	■	■	■	■	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P201	■	■	■	■	■	■	■	■	□	■	■	□	■	■	□	■	■	■	■	□	■	■	■	■
P215m	■	■	□	■	□	□	■	■	□	■	■	■	■	■	■	■	■	■	■	□	■	■	■	■
P208	■	■	■	■	■	□	■	■	□	■	■	□	■	■	■	■	■	■	■	□	■	■	■	■
P200	□	■	■	□	■	□	■	■	□	■	■	□	■	■	□	■	■	□	□	□	□	■	■	■
P203	□	□	■	□	■	□	■	□	□	■	□	□	□	□	□	■	■	□	□	□	□	■	■	■
LEBANESE SOURCES																								
AJ7	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
9N 36(2)	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
57N	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
9N 56(1)	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
IAM-160X	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
IAM-165X	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■
IAM-166X	□	■	■	□	■	□	■	□	□	■	□	■	□	■	□	■	■	□	□	■	■	■	■	■

[z]Filled and empty boxes represent positive and negative reactions, respectively.

can sources tested (except for P203m). Source CPsV-4 is close only to the Spanish source Sp2. These results are in agreement with the behavior of the Mabs previously produced against

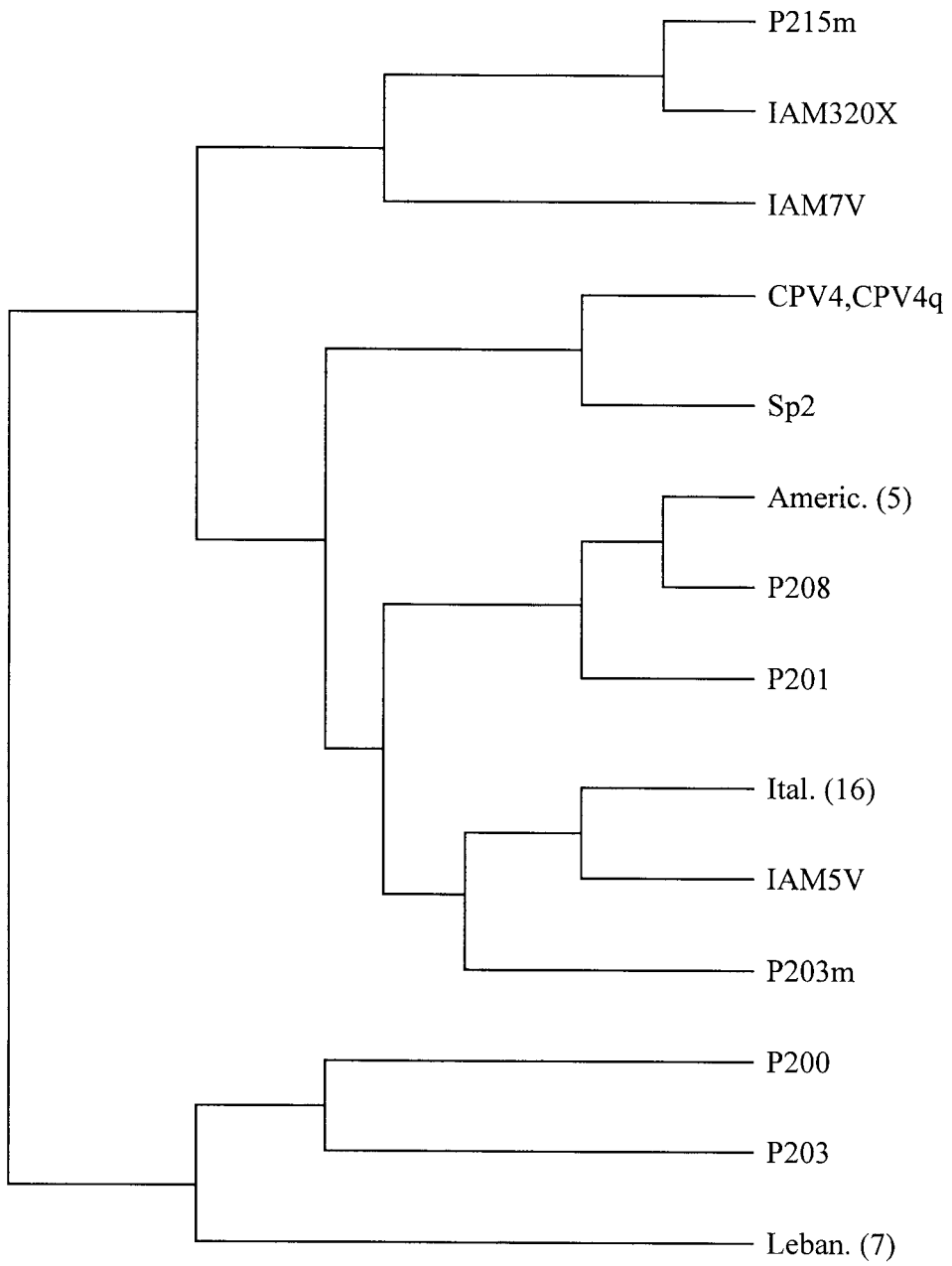

Fig. 1. A dendrogram showing the serological relationships among 40 sources of CPsV, as deduced from the search of 16 epitopes identified in the isolate IAM191Xa.

CPsV-4 isolate, which did not react against any of the other isolates tested (2). This grouping was referred to as "type strain" subcluster.

(V) An additional cluster was composed by two Italian (IAM-320X and IAM-7V) and an American (P215m) sources.

As to the American source P203m, it is worth noting that it originated from a citron plant infected by mechanical transmission (knife cuts) from the citron P203 (9).

As shown in Table 2, source P203 is one of the American virus sources included in the "Lebanese cluster". Surprisingly, P203m behaved differently containing epitopes "I" and "R" which are typically missing in the "American cluster" and in the American sources of the "Lebanese cluster". A possible explanation for this unexpected behavior can be found by admitting that our sample of P203m was infected by a different isolate of CPsV. In any case, it is clear that the American sources provided by C.N. Roistacher seem to be more heterogeneous than other groups, perhaps because they came from a collection that includes samples originating from different areas.

Wide range Mabs. Mabs.PS24, 31, 32 and 34 identified all 40 virus sources analyzed in detail, but did not react with some of the remaining 24 virus sources (data not shown). These sources had been discarded from further analysis because they appeared to have a low virus titre, which made ELISA reactions unreliable. Thus, it cannot be excluded that a higher virus concentration would made some (or perhaps all) of these sources recognizable by the above four Mabs.

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