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Production of monoclonal antibodies against apple proliferation phytoplasma and their use in serological detection

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Abstract

Two monoclonal antibodies were obtained against the apple proliferation phytoplasma that provide easy, rapid, specific and sensitive serological detection. They reacted specifically by using ELISA and immunofluorescence techniques with apple proliferation-infected periwinkles and apple trees from different regions in northern Italy and Slovenia, but not with several other phytoplasma isolates. We did not observe any monoclonal antibody reaction even using phytoplasmas belonging to the same phylogenetic group such as European stone fruit yellows and pear decline. Two serological techniques, immunofluorescence and ELISA, were compared with DAPI staining and PCR. From July until leaf fall ELISA was as sensitive as PCR but was more rapid and convenient than PCR; immunofluorescence was useful for specific detection of apple proliferation phytoplasma on roots throughout the year. Serological techniques could be conveniently applied in the roots, stems and leaves of apple trees depending on specific phenological stages of the plants.

Abbreviations: AP – apple proliferation; DAPI – 4′, 6-diamidino-2-phenyl indole; ELISA – enzyme linked immunosorbent assay; ESFY – European stone fruit yellows; IF – immunofluorescence; mabs – monoclonal antibodies; PCR – polymerase chain reaction; PD – pear decline.

Introduction

Apple proliferation (AP), now known to be a phytoplasma-associated disease, was first reported in Italy in 1950 (Rui et al.), and occurs in many European pome fruit growing areas. Recently, new epidemics of the disease were reported in Italy (Loi et al., 1995; Minucci et al., 1996; Frisinghelli et al., 2000). The causal agent belongs to the phylogenetic 'apple proliferation' group and it is genetically closely related to the agents of pear decline and European stone fruit yellows (Seemüller et al., 1998). The most common symptoms of AP are witches' broom, consisting of small leaves with a short petiole and enlarged stipules, bronze reddish and chlorotic leaves, an abnormal number of petals, and pale, flat fruits with a long petiole. Up to now AP diagnosis is largely based on DAPI (Seemüller, 1976) and molecular biology techniques (Firrao et al., 1994; Lorenz et al., 1995; Rajan et al., 1995). The former technique is rapid, but not specific and sometimes the results obtained can be difficult to interpret. The molecular techniques are specific and highly sensitive, but they are tedious and difficult to employ on a large scale. Serology is also used for phytoplasma detection, though only a few specific polyclonal and monoclonal antibodies (mabs) have been developed against a limited number of phytoplasmas (Lin and Chen, 1985; Jiang et al., 1989; Schwartz et al., 1989; Davies and Clark, 1992). The difficulties encountered in the antigen purification are well known. The great advantage of serological methods is their large-scale utilisation.

The aim of this work was to find an easy, rapid, specific and sensitive method for AP diagnosis. The first step was to obtain mabs against AP phytoplasma. To optimise the diagnosis we tried different serological methods applied to different parts (roots, stems and leaves) of apple trees at different phenological stages and during different seasons. The compared methods were DAPI and PCR.

Materials and methods

Monoclonal antibody production

The partially purified antigen was obtained from AP-infected *Catharanthus roseus* G. Don (periwinkles) plants, following the Jiang and Chen (1987) method. The *C. roseus* test plants were originally inoculated via dodder bridges from symptomatic apple trees (Carraro et al., 1988). One BALB/C mouse was immunised; the spleen and myeloma cell (NSO/1) fusion was diluted in 250 ml of hybridoma selective medium. The grown hybridoma supernatants were screened for the presence of AP phytoplasma-specific antibodies using indirect ELISA with a biotin–avidin system; ELISA plates were coated with partially purified material from healthy and diseased periwinkles (Jiang et al., 1989). The hybridomas that produced antibodies against AP phytoplasma were selected, cultured and submitted to limit dilution for single cell-line selection.

Antibody class and subclass were determined on tissue culture supernatant by indirect ELISA, using the Isotyping Kit II (Pierce Chemical Co., USA), according to the instructions. Cross-sections of leaf midribs and stems were prepared from healthy and AP-infected periwinkles and tested in IF (see below) using hybridoma supernatants. Ascitic fluid was produced by injecting about 10^6 hybridoma cells into pristane primed BALB/C mice. Mabs were purified by affinity chromatography (Immunopure A/G Purification Kit, Pierce Chemical Co., USA).

Specificity of monoclonal antibodies

Indirect ELISA and IF were used to test the specificity of AP mabs (Chen and Jiang, 1988) and to reveal the serological correlation among 38 phytoplasma isolates on *C. roseus* originating from naturally infected herbaceous and woody plants, and belonging to several phylogenetic groups (Bertaccini et al., 2000) (Table 1).

AP detection

Field trees. In September 1999 leaf and stem samples from 500 symptomatic apple trees were collected in Friuli-Venezia Giulia (F-VG) and analysed by using ELISA and IF techniques. The remaining 50 samples, collected from different regions of northern Italy (Trentino and Piedmont) and Slovenia were also subjected to the serological tests. Direct ELISA and IF were applied using both mabs described below. Seven symptomatic apple trees cv. Golden Delicious from an orchard located in F-VG were chosen for a systematic analysis of the colonisation pattern during different phenological stages of the trees. Roots and stems were examined by IF and DAPI techniques and the leaves by direct ELISA and PCR. During an entire year, we repeated eight times the same analyses on organs of the same trees (Table 2). We examined serologically and by using DAPI different symptomatic Japanese plum trees infected by the ESFY phytoplasma and pear trees infected by the PD agent.

Direct ELISA. The plates were coated with 100 µl of the purified mabs 1F4/1E2 and 7H1/2C2 at 5×10^{-3} mg ml^{-1} of coating buffer, for 3 h at 37 °C. The sample preparations consisted in 2 g of fresh midribs of leaves from periwinkles or apple trees, macerated in a mortar with 15 ml of isolation buffer (Jiang and Chen, 1987) and submitted to differential centrifugation (2000 *g* for 10 min and 20,000 *g* for 30 min). The pellets were resuspended in 400 µl of extraction buffer (PBS-Tween 20 0.05%) and 100 µl were dispensed on coated plates and stored overnight at 4 °C. Our preparation of biotin-labelled mabs (Guesdon et al., 1979), 1 : 2000 diluted in conjugate buffer (PBS-Tween 20 0.05% containing Polyvinylpyrrolidone 2%), were added and incubated for 2 h at 37 °C. Streptavidin–alkalin phosphatase conjugate (Boehringer Mannheim no. 1089161), diluted 1 : 5000 in conjugate buffer, was then added to the plates for 30 min at 37 °C. The final steps consisted of enzyme substrate addition (Sigma no. P104); the plates were then read at 405 nm. A different procedure was successively adopted: the two mabs were directly conjugated with alkaline phosphatase and used, diluted 1 : 1000 in conjugate buffer, in DAS-ELISA. The sample preparation was also simplified as follows: the midribs were either

Table 1. Specificity of apple proliferation mabs 1F4/1E2 and 7H1/2C2 undiluted supernatants for a series of *C. roseus* plants individually infected with different phytoplasma isolates belonging to several phylogenetic groups, determined by indirect ELISA and IF. The DAPI test was included for comparison

Phytoplasma isolate (1)	16Sr Group (2)	ELISA (3)	IF (3)	DAPI (3)
AY(4), OS, SA, SIL, TWB, VE	I-B (Aster yellows)	−	−	+
ACH, CA, C-CP, LEO	I-C (Aster yellows)	−	−	+
API, CP, CR, ER, FD, LNI, LNII, MA, RA, TA, TRp, TRr, VAC(4), WX	III-B (X-Disease)	−	−	+
AP, AT(4)	X-A (Apple proliferation)	+	+	+
PD(4)	X-C (Apple proliferation)	−	−	+
LNp, LNS1, LNS2	X-B (Apple proliferation)	−	−	+
A-SLO, BA, BAI, LNIV, P-TV, SA1, SE, SI	XII-A (Stolbur)	−	−	+
Healthy periwinkle		−	−	−

(1) Original source: ACH = *Achillea millefolium*; AP = *Malus communis*; API = *Catharanthus roseus*; A-SLO = *Aster chinensis*; AT = *M. communis*; AY = *A. chinensis*; BA = *C. roseus*; BAI = *C. roseus*; CA = *Daucus carota*; C-CP = *Trifolium* spp.; CP = *T. repens*; CR = *Crepis biennis*; ER = *Erigeron annuus*; FD = *Vitis vinifera*; LEO = *Leontodon hispidus*; LNI = *Prunus salicina*; LNII = *P. salicina*; LNIV = *P. salicina*; LNp = *P. salicina*; LNS1 = *P. salicina*; LNS2 = *P. salicina*; MA = *Chrysanthemum leucanthemum*; OS = *C. roseus*; PD = *Pyrus communis*; P-TV = *Solanum lycopersicum*; RA = *Ranunculus* spp.; SA = *Oxalis acetosella*; SA1 = *O. acetosella*; SE = *Apium graveolens*; SI = *Silene alba*; SIL = *S. vulgaris*; TA = *Taraxacum officinale*; TRp = *T. pratense*; TRr = *T. repens*; TWB = *Tagetes patulus*; VAC = *Vaccinium* spp.; VE = *Veronica arvensis*; WX = *P. persica*.
(2) According to Lee et al. (1998); see also Bertaccini et al. (2000).
(3) + = positive reaction; − = negative reaction.
(4) Kindly provided by Dr. E. Seemüller.

Table 2. Reliability of different diagnostic techniques as influenced by the different plant organ analysed and by the season: seven apple trees (cv. Golden Delicious) showing symptoms of apple proliferation in nature were selected for the test each time

Plant organ analysed	Test compared	Date of analyses, phenological stages of the trees and results obtained (1)							
		Feb. 26 Dormant bud	Apr. 30 Blossom	June 17 Nut-sized fruit	July 28 Final fruit size	Sept. 01 Ripening	Oct. 06 Harvesting	Nov. 10 Leaf fall	Jan. 14 Dormant bud
Root	IF	7/7	7/7	7/7	7/7	7/7	7/7	7/7	7/7
	DAPI	7/7	7/7	7/7	5/7	6/7	7/7	7/7	6/7
Stem	IF	7/7	0/7	3/7	3/7	7/7	7/7	7/7	7/7
	DAPI	7/7	0/7	1/7	2/7	7/7	7/7	6/7	6/7
Leaf	ELISA	n.d. (2)	0/7	4/7	7/7	7/7	7/7	7/7	n.d. (2)
	PCR	n.d. (2)	0/7	5/7	7/7	7/7	7/7	7/7	n.d. (2)

(1) Nominator = no. of plants showing positive to the analyses; denominator = no. of plants tested.
(2) n.d. = test not executed (no leaves present).

directly minced in the mortar or pressed with 15 ml of extraction buffer and then 100 µl of the sap were added to the coated plates.

Immunofluorescence and DAPI staining. One centimetre long pieces of stems and roots were fixed in 4% paraformaldehyde in PBS, and left overnight at 4 °C. They were then longitudinally cut by a cryomicrotome (Leitz Jung 1500) to obtain sections of 20 µm thickness. The sections were treated with the mab tissue culture supernatants and incubated for 1 h at 37 °C. After washing, FITC (fluorescein-isothiocyanate) – antimouse conjugate (Sigma no. F1010) was added and incubated at 37 °C for 30 min. After washing, DAPI stain (1×10^{-3} mg ml^{-1} PBS, Sigma no. D9542) was added and the sections were observed under an epifluorescence microscope. In this way the same section was analysed twice: by IF and by DAPI staining.

PCR/RFLP analyses. DNA was isolated from approximately 1.5 g of leaf petiole and midrib tissues from each sample following the modification of the phytoplasma enrichment procedure developed by Kirkpatrick (Malisano et al., 1996). The presence of the phytoplasmas was determined by PCR using the ribosomal primers f01/r01 (Lorenz et al., 1995). Five microlitre of the PCR products were analysed by electrophoresis in 1.5% agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8) in the presence of 5×10^{-4} mg ml^{-1} ethidium bromide. To distinguish between PD, AP and ESFY phytoplasmas, 10 µl of the PCR products were digested separately with *Ssp*I and *Bsa*AI, according to the manufacturer's instructions (New England BioLabs). Restriction fragments were resolved in a 5% polyacrylamide gel. After electrophoresis, the DNA was stained with silver nitrate.

Results

Monoclonal antibodies production

From the 986 hybridomas obtained and screened by indirect ELISA, four were selected for AP antibody production and submitted to limit dilution. Two monoclonal hybridoma cell-lines (1F4/1E2, 7H1/2C2) were chosen, grown and stored in liquid nitrogen. The mabs 1F4/1E2 and 7H1/2C2 were, respectively, of the IgG1 and IgG2b isotypes and reacted specifically with the AP-infected periwinkle. The ELISA absorbance values after 1 h using the two mabs ranged from 0.015 to 0.025 with healthy plants and from 0.500 to 0.800 with AP-infected periwinkles. The results of immunofluorescence on the cross-sections of midribs from both healthy and AP-infected periwinkles stained with mabs from undiluted hybridoma culture supernatants demonstrated that only sieve tube elements of sections from diseased plants show FITC-specific fluorescence. From ascitic fluid purification we obtained IgG concentrations of 1.1 mg ml^{-1}.

Specificity of the mabs

Table 1 shows the results for the specificity of the mabs 1F4/1E2 and 7H1/2C2, by using indirect ELISA and IF on several phytoplasma isolates maintained in periwinkles. The mabs reacted positively, both in ELISA and IF, only with AP-infected periwinkles, Italian isolate (AP) and German isolate (AT). No reactions were obtained with the other phytoplasmas, belonging to several phylogenetic groups. All the examined plants were shown to be phytoplasma-infected by using DAPI. No positive results were obtained by ELISA, IF and DAPI with negative controls.

AP detection

Direct ELISA was a reliable method for AP diagnosis on leaves from 550 symptomatic apple trees collected in the field in the fall in different areas of northern Italy and Slovenia. The absorbance values using both mabs 1F4/1E2 and 7H1/2C2, 1 h after substrate addition, ranged from 0.600 to 1.100 with AP-infected material; healthy control absorbances ranged from 0.020 to 0.030. No significative differences were observed between the two different mabs employed both in biotin–avidin ELISA and DAS–ELISA. Both sample preparations (sap centrifugation and crude sap) gave similar ELISA results. IF was useful and rapid for AP identification in the phloem of stems of symptomatic trees (Figure 1).

Table 2 shows the results obtained by using different diagnostic techniques on seven symptomatic apple trees cv. Golden Delicious, tested eight times at different phenological stages of the trees during an entire year. Using IF, the presence of AP phytoplasma in the root phloem was observed all year round. For the analyses on stems, IF gave good results from September to February. The detectability of the phytoplasmas using IF was higher than DAPI tests, both on roots and stems. The results obtained from ELISA and PCR were identical from July to November (phenological stage 'leaf fall'); in June (stage 'nut-sized fruit') the sensitivity of PCR was higher than ELISA; in spring (stage 'blossom') both methods gave negative results. Samples from ESFY-diseased Japanese plum and PD-diseased pear trees, analysed using the two AP mabs in ELISA and IF, gave negative results. In contrast, the DAPI test revealed the presence of phytoplasma in the sieve tube elements.

Discussion

In order to gain rapid, specific, sensitive diagnostic techniques for the detection of AP phytoplasma, we obtained two mabs. They belonged to the IgG class; this demonstrated that the mouse was well immunised and the antibody manipulation was easy. In ELISA and immunofluorescence, the two mabs reacted with

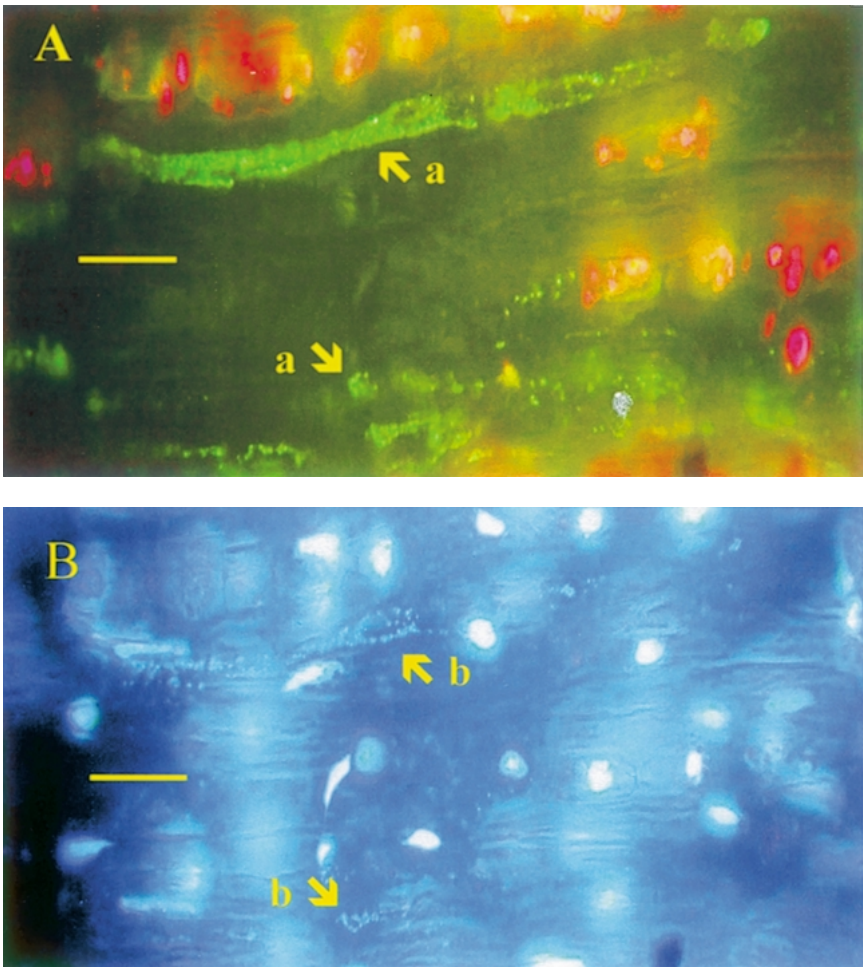

Figure 1. (A) Immunofluorescence staining on longitudinal section of apple proliferation-infected apple tree stems using 1F4/1E2 mab derived from undiluted tissue culture supernatant; arrows (a) indicate typical green FITC fluorescence. (B) Same section as in A, but stained with DAPI; arrows (b) indicate typical DAPI fluorescence. Bars = 50 μm.

AP-infected periwinkles (Italian and German isolates) and with AP-infected samples of apple trees from various regions of northern Italy and Slovenia. The mabs were highly specific; they did not react with other phytoplasma isolates maintained in periwinkles, belonging to several groups. Among these we included isolates of the PD and ESFY phytoplasmas, belonging to the same phylogenetic group as apple proliferation. Similarly the mabs did not react with material of PD-infected pear trees and ESFY-infected Japanese plums. To optimise the diagnosis we applied the IF technique on roots and stems of AP-infected apple trees at different phenological stages, and the ELISA technique on leaves of the same plants, using DAPI and PCR for comparison. On the basis of the results we can affirm that: IF tests on roots can be conveniently applied all year round; IF tests on stems can be conveniently applied from the phenological stage of ripening until the dormant buds stage; ELISA on leaves can be conveniently applied from the end of July until the leaf fall stage. In this experiment the IF technique was found to be more sensitive than DAPI; in fact some samples showed IF positive and DAPI negative for the presence of phytoplasmas. Furthermore, fluorescence in IF was clear and without background. The two techniques, IF and DAPI, can be applied on the same section by changing the microscope filters: in this case it is possible to compare the two methods and at the same time apply two different diagnostic techniques on the same sample. In June the two highly efficient techniques (ELISA and PCR)

did not demonstrate AP infection in any of the seven apple trees. This is probably due to the slow or poor colonisation of the aerial part of the plants at this time of the year. From July to November, when the phytoplasma colonisation of the canopy is high (Seemüller, 1988), the results obtained using ELISA and PCR on the same trees are identical. During this period, the serological technique can substitute for PCR, especially for large-scale diagnosis such as health selection programmes when the number of samples for examination is high. By adopting the DAS–ELISA, the preparation of the samples is made easy and rapid, since DNA extraction is not necessary.

In conclusion, a new tool is now available for the diagnosis of AP phytoplasma. The mabs, recently commercialised by BIOREBA AG (Switzerland), can be conveniently used by applying ELISA and IF; these techniques were found to be easy, rapid, specific and sensitive, especially during particular phenological stages of the apple trees. Research will continue with the aim of obtaining specific mabs against the PD and ESFY agents which cause two other important phytoplasmoses of fruit trees.

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