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Generation and characterization of monoclonal antibodies to Flavescence dorée phytoplasma: Serological relationships and differences in electroblot immunoassay profiles of Flavescence dorée and Elm yellows phytoplasmas

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Abstract

Eleven stable hybridoma cell lines secreting monoclonal antibodies specific for FD-phytoplasma, the pathogenic agent of grapevine Flavescence dorée, were produced by fusing a non-secreting myeloma cell line with spleen cells from Balb/c mice immunized with Flavescence dorée phytoplasma purified by immunoaffinity. These monoclonal antibodies were characterized for their recognition of phytoplasma proteins by western blot. Six of eleven reacted specifically in ELISA and immunoblotting with Elm-yellows phytoplasma. These antibodies did not react either in ELISA or in western blot with preparations from periwinkles infected with phytoplasmas that cause GYU (Grapevine Yellows from Udine), AP (Apple Proliferation), EAY (European Aster Yellows) and StolC (Stolbur from France). Two of these hybridoma lines were used routinely for the immunodiagnosis of Flavescence dorée phytoplasma in diseased grapevines.

Abbreviations: ELISA – Enzyme linked immunosorbent assay; FD – Flavescence dorée; MLO – Mycoplasmalike organism; EY – Elm yellows.

Introduction

Phytoplasmas (formerly Mycoplasma-like organisms, MLO) are wall-less prokaryotes grouped in the class Mollicutes that are thought to be the causal agents of more than 200 diseases of higher plants (McCoy et al., 1989; Kirkpatrick, 1989). Because of the inability to isolate phytoplasmas in pure culture, a formal taxonomy for these pathogens has never been established. Moreover, access to the constituents of phytoplasma cells is limited and in particular little information is available about the number, the nature and the role of the membrane proteins.

Grapevine Flavescence dorée (FD) is an important yellows disease of grapevine in France (Caudwell and Ottenwaelter, 1957; Caudwell, 1990). A phytoplasma has been demonstrated to be the etiological agent of the disease (Caudwell et al., 1971). The natural vector of FD is the leafhopper *Scaphoideus littoralis* Ball (Schvester et al., 1962; Caudwell et al., 1970), which transmits the pathogen agent by phloem feeding according to the persistent mode. For laboratory studies, FD-phytoplasma has been maintained in broadbean (*Vicia faba* L.) under greenhouse conditions, using the leafhopper *Euscelidius variegatus* Kbm for transmission from broadbean to broadbean (Caudwell et al., 1972).

Antisera to FD-phytoplasma have been produced (Boudon-Padieu et al., 1989) but thorough cross absorption was needed to get rid of antibodies to host components. However, western blots with polyclonal antibodies raised to phytoplasma-enriched fractions of FD-infected broadbean have demonstrated the presence in FD-infected leafhoppers of two main and 7–10 minor components which were not detected in healthy leafhopper control (Boudon-Padieu et al.,

1987; Seddas et al., 1993). Specific monoclonal antibodies (Köhler and Milstein, 1975) have the advantage over polyclonal antibodies to avoid cross reactivity against host material. For this reason, monoclonal antibodies to FD-phytoplasma enriched preparations, which had been obtained in several fusions (Schwartz et al., 1989), were reacted on western blot of proteins prepared from FD-infected leafhopper or broadbean (Seddas et al., 1993; Seddas et al., 1995). Labeling was obtained only on either of the two main components and not on any of the minor components revealed with polyclonal antibodies. These results might indicate either that some of the phytoplasma antigens had been destroyed or lost in the preparation prior to immunization of mice, or that they were in too low titres to induce elicitation of homologous antibodies in the animals, or else that hybridomas secreting these antibodies were too few and had escaped screening (Clark et al., 1989).

In a recent publication, Seddas et al. (1993) have described a method for the purification by immunoaffinity of larger quantities of FD-phytoplasmas. The immunoaffinity-purified phytoplasma cells are undamaged and the morphology of the phytoplasma structure following this procedure was reported well preserved (Seddas et al., 1995).

Advantage was taken to produce monoclonal antibodies to these purified phytoplasmas, and to explore their reactivities by western blots of proteins of purified phytoplasmas. In addition, serological relationships were investigated with several phytoplasmas, including elm yellows phytoplasma which belongs to the same ribosomal cluster as FD (Schneider et al., 1993), and can be detected with non-ribosomal primers for PCR constructed from FD DNA (Daire et al., submitted).

Another aim of monoclonal antibody production is to obtain efficient and sensitive tools for phytoplasma diagnosis on diseased plants (Lin and Chen, 1985; Clark et al., 1989; Sarindu and Clark, 1993; Garnier et al., 1990). Grapevine is a difficult plant for phytoplasma diagnosis. Though ELISA detection of FD in diseased grapevine has previously been obtained (Caudwell and Kuszala, 1992), monoclonal antibodies from former fusions which were very efficient in ELISA on leafhopper and herbaceous hosts were deceiving in their use on infected grapevine tissues in ELISA and immunoelectron microscopy (C. Kuszala and J. Lherminier, personal communications).

In this paper, we report the production and characterization of monoclonal antibodies obtained from mice immunized with purified and preserved FD-phytoplasmas. They were highly reactive in ELISA of FD-diseased grapevine. They demonstrated serological relatedness though differences in antigens on western blots of FD and EY phytoplasmas.

Materials and methods

Phytoplasma sources

Healthy and FD-infected *E. variegatus* leafhoppers were reared as previously described (Caudwell and Larrue, 1977). FD-infected leafhoppers were obtained by feeding young adults on FD-diseased *V. faba* for 3 weeks (feeding access period), then on healthy *Zea mays* L. Infected leafhoppers were used after 8 weeks following the beginning of feeding access, when the phytoplasma titer is high in their body (Boudon-Padieu et al., 1989). Other phytoplasmas were maintained in periwinkle. **GYU** (Grapevine Yellows from Udine) was dodder-transmitted from grapevine to periwinkle by Dr. Osler, University of Udine, Italy; **AP** (Apple Proliferation) from Dr. Carraro, University of Udine, Italy; **EAY** (European Aster Yellows) originated from Dr. Marwitz, Germany; **EY** (Elm Yellows from New York) was isolated by W.A. Sinclair; **StolC** (Stolbur from France) was isolated by Dr. M.T. Cousin, Versailles, France. In addition, the **FD** source for ELISA was FD70 transmitted to periwinkle by leafhoppers in our laboratory. Healthy periwinkles were used as controls in ELISA.

Preparation of antigens for ELISA

Leafhoppers. Healthy and FD-infected leafhoppers were individually crushed with a glass rod in a final volume of 500 μl PBS (phosphate-buffered saline: 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) (Boudon-Padieu et al., 1989). The supernatant of a 2,900 g $\times$ 5 min centrifugation was used for ELISA (100 μl per well).

Grapevines. Fresh leaves of healthy and FD-diseased grapevines were crushed using a mortar in grinding buffer (0.25 M Tris, 0.8% NaCl, 0.5% ascorbic acid, 4% Polyclar AT, 5% Chaps, 0.02% Thimerosal, pH 8.2; 1 g per 10 ml) (Caudwell and Kuszala, 1992). This brei was centrifuged at 2,900 g for 5 min and the supernatant was used for ELISA (100 μl per well).

Periwinkles. Extracts from leaves of infected periwinkle (1 g fresh weight per 10 ml of PBS, 10 mM β-mercaptoethanol, pH 7.6) were filtered through 100 μm nylon mesh and clarified by centrifugation (2,900 g for 10 min). Healthy periwinkle extracts were used as control.

Immunogen preparation

The procedure for immunoaffinity purification for FD-phytoplasmas from leafhoppers has been described by Seddas et al. (1993). Briefly, after loading of the column with a phytoplasma enriched preparation in 1464 medium (Caudwell and Kuszala, 1986), and washing with 0.025 M Tris-base, 0.15 M NaCl, pH 7.4, phytoplasmas were eluted with 0.1 M glycine buffer pH 11.5. The eluate was monitored at OD_{280} nm and 5 ml fractions were collected. The elution fraction containing phytoplasma antigens was centrifuged at 40,000 g for 30 min. The supernatant was then discarded and the pellet was resuspended in 0.5 ml of PBS for immunization. Protein was estimated routinely by the dye-binding method of Bradford (1976) using BSA (Bovine Serum Albumin) as a standard.

Preparation of monoclonal antibodies

Six-week-old female Balb/C mice were injected intraperitoneally with 0.1 ml of purified phytoplasma preparation diluted in PBS (100 μg protein) after mixing with 0.1 ml of Freund's complete adjuvant (GIBCO, BRL). Two booster intraperitoneal injections using preparations emulsified in incomplete adjuvant were made at 2-week intervals. One week after the third injection, mice were bled by tail and the sera were tested by ELISA against FD-infected and healthy leafhoppers. When the bleed was positive at 10,000 fold serum dilution, the mice were boosted with 100 μg of phytoplasma antigens in PBS. Mouse spleen cells were fused with myeloma cells (line SP2/0-Ag 14) essentially as described by Köhler and Milstein (1975). Four days later, spleen cells were harvested and fused with Sp2/O myeloma cells at a ratio of 10:1 using polyethylene glycol (PEG, MM-1500, Boehringer). The fusion product was distributed in five 96-wells tissue culture plates pre-coated with peritoneal macrophages diluted in 0.1 ml of complete HAT (Hypoxantine-Aminopterin-Thymidine) selective medium which supports the growth of spleen-myeloma hybrid cells, but not that of non-fused myeloma cells or myeloma-myeloma cell hybrids (Köhler and Milstein, 1975).

Screening and cloning of hybridomas

A DAS-ELISA was used to screen the hybridoma cells secreting specific antibodies, according to standard ELISA procedures (Schwartz et al., 1989). After ELISA screening, hybridomas that secreted antibodies reacting with FD-infected leafhoppers but not with healthy leafhoppers were selected for further cloning by limit dilution (Goding, 1980). The positive clones were subcultured twice to obtain stable monoclonal hybridomas. Monoclonal hybridoma cells were subcultured for antibody production and stored in liquid nitrogen for further use.

Subtyping of monoclonal anti-FD-phytoplasma antibodies

The subclass and light chain type of the monoclonal antibodies were determined from the hybridoma medium using a test kit from GIBCO, BRL.

Ascitic fluid production

Ascitic fluid containing monoclonal antibodies was produced in pristane-primed Balb/c mice by injecting approximatively 10^6 hybridoma cells into the peritoneal cavity and then collecting ascitic fluid after 2 to 3 weeks (Hoogenraad et al., 1986). Monoclonal antibodies were obtained from ascitic fluid using an IgG affinity purification kit with protein A (Pierce) (Seddas et al., 1993).

SDA-PAGE and immunoblotting

After clarification by centrifugation (2,900 g for 10 min), total protein content of diseased periwinkle extract was precipitated for 2 h with 10% (w/v) TCA at 4 °C. The pellet obtained after centrifugation (10,000 g for 10 min) was washed with 1 ml acetone 100% for a period of 1 h at −20 °C. After centrifugation (10,000 g for 10 min), the pellet was resuspended in 50 μl of water, 62.5 μl of protein solubilization buffer (0.5 M sucrose, 0.1 g l^{-1} Bromophenol blue, 5 mM EDTA, 10 g l^{-1} methionine, 0.2 M Tris-HC1, pH 6.8), 6 μl of 20% (w/v) SDS and 1 μl of 1 M dithiothreitol.

After denaturation, proteins obtained from disease periwinkle and proteins of purified FD-phytoplasma were separated on a 12% denaturing polyacrylamide gel by SDS-PAGE according to the method of Laemmli (1970). Proteins were transferred from the polyacrylamide gel by electroblotting onto Immobilon-P™ (Millipore) membrane using a Millipore semi-dry transfer apparatus according to the manufacturer's instructions. After proteins being transferred, the

Table 1. Differential immune reactivity of monoclonal antibodies with extracts from leaves of grapevine or from leafhoppers using double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA)

Cell line	Antibody isotype	ELISA			
		Leafhopper		Grapevine	
		Infected	Healthy	Diseased	Healthy
2C5A7	IgG3	1.732	0.021	1.120	0.040
3A1H3	IgG2b	1.820	0.015	1.287	0.055
3A1G7	IgG2b	1.620	0.027	1.131	0.024
2C10B2	IgG1	2.100	0.013	0.807	0.046
2D10G1	IgG2b	1.530	0.039	1.210	0.013
2C10F5	IgG2a	3.900	0.092	0.718	0.043
2A1G5	IgG2b	0.550	0.060	1.045	0.033
5C7	IgG2b	1.603	0.045	1.216	0.028
2D10C1	IgG2b	1.210	0.032	0.947	0.059
2F5	IgG1	2.130	0.040	1.250	0.019
6D9	IgG1	1.862	0.039	1.384	0.027

membranes were treated with the immunological method described previously for the detection of FD-phytoplasma antigens in broadbean (Seddas et al., 1995).

Results

Cell fusion and hybridomas selection

The fusion was carried out with splenocytes obtained from a mouse immunized with FD-phytoplasma antigens immunopurified from infected leafhoppers. At the stage of the first limit dilution, a total of 98 wells containing hybridoma clones reacted positively in ELISA against FD-phytoplasma infected leafhoppers. All wells containing hybridoma clones showed a negative ELISA response using healthy leafhopper antigens. Eleven stable hybridomas were chosen for subcloning and further characterization of monoclonal antibodies.

ELISA response

The ELISA values of the eleven selected monoclonal antibodies on leafhopper extracts and on grapevine extracts are shown in Table 1. All of them reacted with high values on diseased grapevine.

Monoclonal antibodies 2F5 and 6D9 which displayed consistent positive ELISA values on FD-diseased grapevine from various infected vineyards were produced in ascitic fluid and IgG purified for routine diagnostic of grapevine yellows. Table 2 shows the ELISA values obtained with monoclonal antibodies to several phytoplasmas. Positive reaction was obtained only with EY-phytoplasma for 6 out of 11 hybridoma supernatants.

Characterization of monoclonal antibodies

Results from immunoblot detection of SDS-PAGE separated and transferred proteins of FD-leafhoppers are presented.

Figures 1a and 1b show the specificity of diverse monoclonal antibodies to the FD-phytoplasma antigens as compared to the rabbit polyclonal anti-FD-phytoplasma antibodies. Rabbit polyclonal antibodies detected several bands of relative molecular weight of 55 kDa, 28 kDa, 20.5 kDa and 19 kDa and a few minor components in positive control lanes a and j. Monoclonal antibodies 6D9 and 2F5 reacted with a 20.5 kDa polypeptide whereas monoclonal antibodies 2D10C1, 2A1G5 and 5C7 recognized a 19 kDa polypeptide (Figure 1a). Monoclonal antibodies 2C10F5, 2C10B2 and 2D10G1 recognized a higher molecular weight polypeptide of 28 kDa (Figure 1a). Monoclonal antibodies 3A1G7, 3A1H3 and 2C5A7 all recognized the same 55 kDa polypeptide (Figure 1b).

The hybridoma supernatants that had reacted positively with EY-phytoplasma in ELISA were assayed on western blots of proteins of EY-infected periwinkle and compared to rabbit anti-FD polyclonal antibodies. Figure 2 shows that several bands of 55 kDa, 42.7 kDa, 38 kDa, 33 kDa, 27.5 kDa, 25.5 kDa and few minor components were labeled by either the rabbit polyclonal antibodies anti-FD (lane a) and the monoclonal antibodies (lanes b–g). Monoclonal antibody 3A1G7 reacted with a 55 kDa polypeptide. Monoclonal antibodies 2D10C1, 2A1G5 and 5C7 all recognized the same 38 kDa polypeptide. Monoclonal antibodies 2F5 and 6D9 were directed against a 20.5 kDa polypeptide.

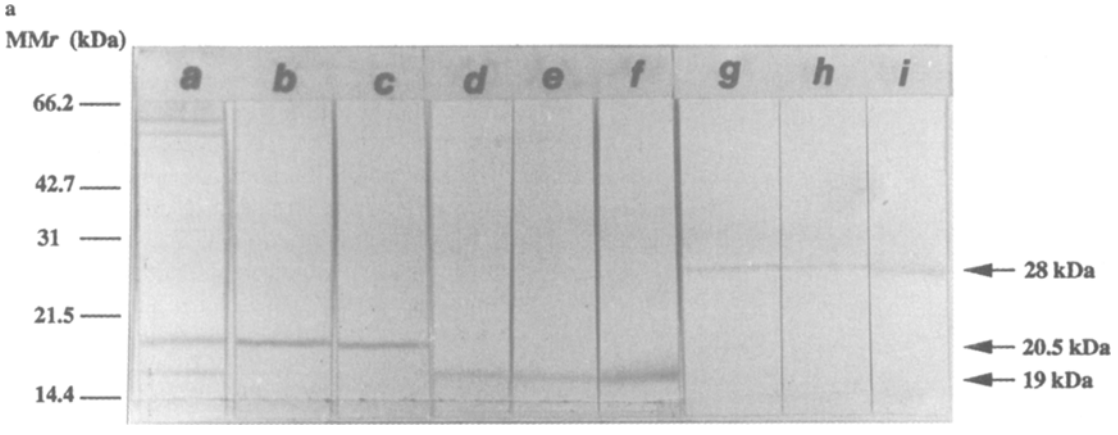
The Table 3 shows the comparative responses obtained in western blot on the FD and EY phytoplasma polypeptides using the different monoclonal antibodies. A peptide of 55 kDa is detected on both phytoplasmas by a single monoclonal antibody (3A1G7). The other monoclonal antibodies react on peptides of different sizes: 38 kDa and 27.5 kDa on EY instead of 19 kDa and 20.5 kDa on FD respectively. The 19 kDa polypeptide in FD does not appear in EY.

Table 2. Detection by ELISA of diverse phytoplasmas using monoclonal antibodies. The antigen preparations obtained from periwinkles were incubated in a microplate. The different monoclonal IgGs were then deposited, followed by anti-mouse IgG alkaline phosphatase conjugate. $A_{405\,nm}$ values were recorded 1 h after substrate addition.

– The $A_{405\,nm}$ values ranged from 0.028 to 0.047 for healthy periwinkle.
– The range values as positive control FD was from 0.5 to 1.4.
– The $A_{405\,nm}$ values obtained ranged from 0.3 to 1.2 for EY-infected periwinkle.

HP (Healthy Periwinkle), **GYU** (Grapevine Yellows from Udine), **AP** (Apple Proliferation), **EAY** (European Aster Yellows), **EY** (Elm Yellows from New-York), **StolC** (Stolbur from France)

Antibodies	HP	FD70	GYU	AP	EAY	EY	StolC
2C5A7	–	+	–	–	–	–	–
3A1H3	–	+	–	–	–	–	–
3A1G7	–	+	–	–	–	+	–
2C10B2	–	+	–	–	–	–	–
2D10C1	–	+	–	–	–	+	–
2C10F5	–	+	–	–	–	–	–
2A1G5	–	+	–	–	–	+	–
5C7	–	+	–	–	–	+	–
2D10C1	–	+	–	–	–	–	–
2F5	–	+	–	–	–	+	–
6D9	–	+	–	–	–	+	–



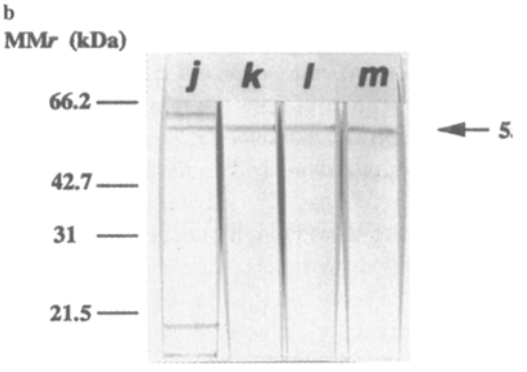


Figure 1a and b. Western-blot identification of FD-antigens recognized by the anti-FD monoclonal antibody-containing supernatants. Proteins of immunoaffinity-purified FD phytoplasma were separated in SDS-PAGE and western-blotted onto Immobilon-P^{TM} membrane. Membrane strips corresponding to electrophoresis sample lanes were obtained and incubated with anti-FD antibodies. Lane a and j, rabbit polyclonal antibodies 5 μg/ml in TBST. Other lanes, monoclonal antibody-containing supernatants: lane b, 6D9; lane c, 2F5; lane d, 2D10C1; lane e, 2A1G5; lane f, 5C7; lane g, 2C10F5; lane h, 2C10B2; lane i, 2D10G1; lane k, 3A1G7; lane l, 3A1H3; lane m, 2C5A7. The position of molecular weight markets are indicated on the left.

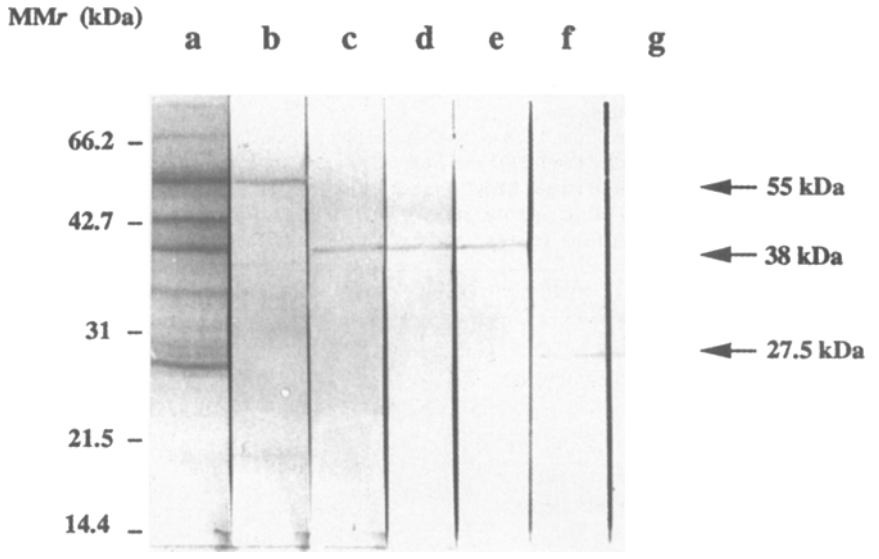


Figure 2. Western-blot of proteins of EY-infected periwinkle with anti-FD monoclonal antibody-containing supernatants. Total proteins of phytoplasma-enriched fraction of EY-infected periwinkle were separated in SDS-PAGE and western-blotted with different anti-FD antibodies. Lane a, rabbit polyclonal antibodies 5 μg/ml in TBST. Other lanes, monoclonal antibody-containing supernatants: lane b, 3A1G7; lane c, 2D10C1; lane d, 2A1G5; lane e, 5C7; lane f, 2F5; lane g, 6D9. The position of molecular weight markers are indicated on the left.

Table 3. FD and EY phytoplasma polypeptides labeled by monoclonal antibodies. Relative molecular masses are expressed in kDa. **FD**: Flavescence dorée; **EY**: Elm Yellows

Antibodies	FD	EY
2C5A7	55	–
3A1H3	55	–
3A1G7	55	55
2C10B2	28	–
2C10F5	28	–
2D10G1	28	–
2F5	20.5	27.5
6D9	20.5	27.5
2D10C1	19	38
2A1G5	19	38
5C7	19	38

Discussion

Phytoplasmas are wall-less prokaryotes which are restricted to phloem tissues in infected plants and specifically transmitted in the multiplying persistent mode by leafhopper species. Little is known about the relations between phytoplasma and their hosts and in particular on the role of membrane proteins in recognition mechanisms (Lefol et al., 1994). In the preceding years, efforts have been made to obtain specific antibodies for sensitive diagnosis and the exploration of phytoplasma route in their host. Because of their unculturability and because of the difficulty to prepare pure immunogens of phytoplasmas, different methods for their extraction have been used in order to produce polyclonal and/or monoclonal antibodies (Lin and Chen, 1985; Boudon-Padieu et al., 1989; Clark et al., 1989; Jiang et al., 1989a; Schwartz et al., 1989; Garnier et al., 1990; Hsu et al., 1990; Fos et al., 1992; Sarindu and Clark, 1993; Shen and Lin, 1993). In most cases, monoclonal antibodies were obtained to a single polypeptide among the phytoplasma proteins (Clark et al., 1989; Jiang et al., 1989b). Yet, in the case of FD, polyclonal antibodies had demonstrated multiple antigens associated to infection in leafhopper and plant hosts (Boudon-Padieu et al., 1987; Seddas et al., 1993). The obtention of monoclonal antibodies homologous to the different epitopes would be valuable for the comparison of phytoplasma strains and isolates, as well as for use in the characterization of membrane proteins which might play a role in the host-pathogen specificity.

Previous work showed that monoclonal antibodies obtained to enriched fractions of infected hosts would label two of these antigens (Seddas et al., 1993 and 1995). These polypeptides were shown by immunosorbent electron microscopy to belong to membrane proteins and one of the monoclonal antibodies was used

to purify by immunoaffinity phytoplasma cells which had retained their physical and biological integrity (Seddas et al., 1993 and 1995).

The present work using immunopurified phytoplasmas to raise antibodies in mice has resulted in the obtention of antibodies specific to two protein components of FD-phytoplasma in addition to the two components already detected (Seddas et al., 1995). The present data confirm that purified phytoplasma have kept minor epitopes in good state and in titres high enough for elicitation of homologous antibodies. On FD antigens western blots, the bigger peptide of 55 kDa could be a dimer or a trimer of each of the two smaller peptides of 28 and 19 kDa. However, as formerly pointed out (Seddas et al., 1993) it is noticeable that each monoclonal antibody labels only one peptides. Thus it seems that the higher molecular weight peptide does not contain the epitopes labeled on the smaller ones. Further screening of the specificity of other hybridoma clones obtained in the fusion might bring more informations on the components of the phytoplasma membrane.

The data presented also show for the first time a serological relationship between FD and EY which belong to the same ribosomal cluster, but are different on the basis of RFLP of genomic fragments (Daire et al., 1992). However, the western blot obtained with FD antibodies on EY antigens shows several differences between FD and EY. Several monoclonal antibodies do not react and most of he others label peptides of different sizes in FD and EY. The possibility that the 38 kDa detected on EY by three monoclonal antibodies is a dimer of the 19 kDa that they label in FD cannot be ruled out. Further experiments on different EY type phytoplasmas should be done to help in serological characterization of isolates in the EY cluster. The possible role of membrane proteins in the specificity of the leafhopper transmission should be investigated. These monoclonal antibodies are precious tools for a further work aiming to a thorough characterization of phytoplasma constituents. In addition, they will be evaluated for diagnosis in grapevine using extraction protocols which could avoid the use of detergents.

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