European Journal of Plant Pathology **103:** 767–774, 1997.
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Monoclonal antibodies for detection, serological characterization and immunopurification of grapevine fleck virus

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Accepted 30 June 1997

Key words: diagnosis, hybridoma, TiterMax^{TM}, Freund

Abstract

Ten stable hybridoma cell lines secreting monoclonal antibodies to grapevine fleck virus (GFkV) were selected after fusing spleen cells of immunized Balb/C mice with mouse myeloma cells (SP2/0-Ag 14). All MAbs reacted positively in ELISA with leaf extracts from fifty GFkV-infected grapevines from various geographical origins. MAb 2B5 was used for routine detection of GFkV and appeared to be more sensitive than polyclonal antibodies. The first attempt to purify GFkV by immunoaffinity chromatography using MAb 2B5 led to highly purified coat protein. This procedure encompassed fewer steps and allowed the use of tissues other than rootlets for satisfactory purification.

Abbreviations: ELISA – Enzyme Linked Immunosorbent Assay; DAS-ELISA – double antibody sandwich ELISA; TAS-ELISA – triple antibody sandwich ELISA; GFkV – grapevine fleck Virus; MAb – monoclonal antibody; PBS – phosphate buffered saline.

Introduction

Grapevine fleck virus (GFkV), characterized by Boulila et al. (1990), was shown to be the agent responsible for the grapevine fleck disease (Boscia et al., 1991). The inhibitory effect of GFkV on the development of roots and the graft take of *Vitis rupestris* was shown to be highly significant (Triolo and Materazzi, 1986). Grapevine fleck disease is included in certification schemes for sanitary selection in different viticultural countries. This points out the need for reliable and easy to use detection methods of GFkV.

Indexing on *Vitis rupestris* du Lot by classical grafting of woody cuttings needs a 3-year observation period in the nursery (Hewitt et al., 1972; Martelli, 1993). The use of green grafting can reduce indexing time to a few weeks (Mink and Parsons, 1977; Walter et al., 1990), but still requires a lot of work and expensive facilities. ELISA methods have been developed and shown to be efficient for GFkV detection in *Vitis vinifera* and American rootstocks (Boscia et al., 1991; Walter and Cornuet, 1993).

However, the production of polyclonal sera against GFkV proved to be difficult because of the low efficiency of virus purification (Boscia et al., 1993; Ramel et al., 1993). This is due to the low concentration of virus located in phloem tissues and to difficulties encountered during purification. The need for grapevines free from other known spherical viruses and for large quantities of young roots for purification are additional difficulties. To overcome these problems, production of monoclonal antibodies (MAbs) was undertaken (Boscia et al., 1993; Ramel et al., 1993; Boscia et al., 1995).

In this paper, we describe an alternative method for rapidly obtaining MAbs using low quantities of antigen. These MAbs were used for detection of GFkV in grapevines, for serological comparison of GFkV isolates and for purification of GFkV based on immunoaffinity chromatography.

Material and methods

Virus sources

The virus source for GFkV purification was a *Vitis vinifera* cv. Muscat d'Alexandrie plant originating from Tunisia. This plant was also infected with grapevine leafroll-associated virus 3 (GLRaV-3) and grapevine virus A (GVA) as confirmed by ELISA. Healthy samples used for ELISA were from the same variety after removing the three viruses by heat treatment (Bass and Legin, 1981). Young roots used for ELISA detection were from healthy and infected grapevines grown in perlite in a glasshouse. The plant sap for ELISA was obtained as described by Walter and Cornuet (1993). Grapevines used for the comparison of GFkV isolates were kindly provided by the laboratories of G.P. Martelli (University of Bari), V. Padilla (C.R.I.A. Murcia) and U. Prota (University of Sassari), or were originated from the collections of ENTAV (Le Grau du Roi) or INRA (Colmar and Montpellier).

Virus purification

Virus purification was essentially as described by Boulila et al. (1990). Rootlets from fast-growing green cuttings were ground in 5 vol. extraction buffer. Enzymatic maceration was done overnight at room temperature after addition of 0.3% (w/v) pectinase (Merck) and 1.5% (w/v) cellulase TC (Merck). Self-forming density gradients (25% sucrose in 0.02 M citrate buffer pH 6.1) were run for 165 min at 24,000 rpm in a Beckman SW28 rotor at 5 °C. Particles were collected using an ISCO fractionator, pelleted by ultracentrifugation at 250,000 g for 2 h at 5 °C then resuspended in PBS buffer. The concentration of protein antigen was estimated by the dye-binding method of Bradford (1976).

Immunization

Six-week-old female Balb/C mice were immunized by intraperitoneal injections using either Freund's adjuvant (GIBCO, BRL) or TiterMax^{TM} adjuvant (Interchim). Two mice were used for each protocol. When using Freund's adjuvant, the first injection contained 50 μg antigen mixed with 1 vol. Freund's complete adjuvant. Two other injections with 50 μg antigen each mixed with Freund's incomplete adjuvant (v/v) were given at 2-week intervals. Seven days after the third injection, the mice were bled by tail and the antisera titers were evaluated. One mouse from each procedure with an antibody titer higher than 1: 10,000 was boosted once with 50 μg GFkV antigen without adjuvant. The fusion was realised three days after the boost.

When using TiterMax^{TM} adjuvant, one injection containing 30 μg antigen plus TiterMax^{TM} (v/v) was made. The titer of the polyclonal antiserum was estimated after 15 days and a boost injection with 30 μg antigen was given.

Preparation of monoclonal antibodies

Mouse spleen cells were fused with myeloma cells (line SP2/0-Ag 14) according to Köhler and Milstein (1975). The fusion product was distributed in five 96-wells tissue culture plates pre-incubated with peritoneal macrophages as described by Seddas et al. (1996).

Screening and cloning of hybridomas

DAS-ELISA was used to screen hybridoma cells secreting GFkV-specific antibodies according to standard procedures (Clark and Adams, 1977). After ELISA screening, hybridoma cells secreting antibodies specific for infected grapevine were selected for further cloning (Schwartz et al., 1989). Hybridoma cells secreting GFkV-specific MAbs were subcloned twice by the limiting dilution method, tested again for antibody production and stored in liquid nitrogen.

Isotyping of the monoclonal antibodies was done on culture supernatants using a murine monoclonal antibodies isotyping kit (GIBCO, BRL).

Ascitic fluid production

High titered ascitic fluids were produced in pristan-primed Balb/C mice by injecting approximatively 10^6 hybridoma cells into the peritoneal cavity. The ascitic fluid was collected through a 19-gauge needle after 2 weeks (Hoogenraad and Wraight, 1986). MAbs were purified from ascitic fluids by affinity chromatography on Ultralink immobilized protein A columns (Pierce).

Preparation of affinity column

Thirty milligrams of purified 2B5 monoclonal antibodies were oxidized and coupled to 10 ml Hydrazid Avidgel (Pierce) (Seddas et al., 1993). Two hundred gram of GFkV infected leaves was processed through the purification protocol till the polyethyleneglycol precipitation (Boulila et al., 1990). The pellet was

dissolved in 30 ml phosphate buffer. After centrifugation of 10 min at 8,000 g, the supernatant was filtered through a 220 nm Syrfil MF filter (Costar). The clear filtrate was considered as the virus enriched fraction and was loaded onto the column. The eluate was monitored at 280 nm with a spectrophotometer coupled to the end of the column. After two successive passages of the same virus fraction, the column was intensively washed with a Tris buffer (0.025 M Tris-HCl pH 7.4, 0.15 M NaCl) until the absorbance at 280 nm decreased to zero. Elution was performed with 0.1 M glycine, pH 11.5 (Seddas et al., 1993). Fractions of 1 ml were collected and GFkV was detected by TAS-ELISA (using MAb 2B5).

ELISA detection of GFkV in infected grapevines

Five different ELISA protocols were compared (Table 3). GFkV polyclonal IgGs were used at a concentration of 0.25 μg ml^{-1} for the coating step and 0.125 μg ml^{-1} when biotinylated. MAb 2B5 was diluted to 2 μg ml^{-1} for coating and 0.25 μg ml^{-1} when used in conjugation with biotin. Protein A (Pharmacia Upsala) was used in saturating conditions (100 μg ml^{-1}). Alkaline phosphatase conjugated goat anti-mouse IgGs (H+L) (Biosys) were used at 0.125 μg ml^{-1}. Streptavidine alkaline phosphatase (Jackson) was used at 0.05 μg ml^{-1}. Substrate was p-nitrophenylphosphate (Boehringer) at 1 mg ml^{-1} in 10 mM diethanolamine pH 9.6. All washings were done with 200μl Tris-casein buffer (10 mM Tris-HCl, 154 mM NaCl, 0.5% (w/v) casein, pH 7.6) and a saturation step using 200μl same buffer (1 h at 37 °C) was always done before antigen incubation. Antigen was incubated overnight at 4 °C, whereas incubations of protein A or antibodies were for 2 h at 37 °C.

Immunoblotting

Purified virus samples were analysed on a 12% sodium dodecyl sulfate acrylamide gel according to Laemmli (1970). Proteins were transfered from the polyacrylamide gel by electroblotting onto an Immobilon-P^{TM} membrane (Millipore) using a Millipore semi-dry transfer apparatus (80 V, 2.5 mA cm^{-2} for 40 min at room temperature) according to the manufacturer's instructions. Non specific protein binding sites were saturated using 2% (w/v) Tween 20 in PBS buffer for 15 min at room temperature. The membrane was incubated for 2 h with either culture supernatants (diluted 5 times in PBST = PBS + 0.05% (w/v) Tween 20) or ascitic fluids (diluted 1,000 times in PBST). After three washings in PBST (5 min each), alkaline phosphatase-conjugated goat anti mouse IgGs (H+L) (Biosys) were added (0.0625 μg ml^{-1} in PBST) for 2 h at room temperature. The immunoblots were washed in PBST as before and then in 100 mM Tris-HCl pH 8.6. Blots were finally developed using 1 mg (per 5 ml) alpha-naphthyl acid phosphate, 0.04% (w/v) fast blue RR salt [4-benzoylamino-2, 5 dimethoxybenzene diazonium chloride hemi (zinc chloride) salt], and 10 mM $MgCl_2$ in 80 mM Tris-HCl pH 8.6.

Immunoelectron microscopy

Pioloform-coated grids were sequentially floated on drops of purified virus suspension, MAb 2B5 (50 μg ml^{-1}) and 5-nm-gold labelled goat anti-mouse IgGs (1:50 dilution) (British Biocell International). All steps were for 30 min and separated by three washes with 10 mM phosphate (Na-K) buffer pH 7.4. After a last wash for 1 min on water, grids were stained with 2% aqueous uranyl acetate before observation on a Phillips EM 208 electron microscope.

Results

Virus purification for immunization

The virus purification yielded 700 μg of antigenic protein from 500 g of rootlets. Observation in the electron microscope revealed spherical particles with negative stain inside with little contamination by cell constituants.

Hybridoma selection

The immunization procedure using Freund's adjuvant gave 260 positives out of 300 in the first screening after 40 days and using 200 μg antigen. The TiterMax^{TM} protocol gave 34 positives out of 64 after 18 days and using 60 μg of antigen.

At the stage of the second limit dilution, four stable hybridomas (2B5, 2F1, 3A4, 3D4) from fusion 1 (Freund's adjuvant) and six stable hybridomas (6A2, 6A11, 6C2, 9 E11, 10B4, 10C1) from fusion 2 (TiterMax^{TM}) were still growing and secreting specific antibodies in continuous culture. Eight of these MAb lines were only used as cell culture supernatants; the other two (2B5 and 3A4) were used as cell culture supernatant and in ascitic fluids (Table 1). In this case the yield of purified

Table 1. Properties and source of the MAbs selected

Fusion	MAb	Isotype	Source of MAb	ELISA titer	
				Direct protocol	Indirect protocol
1	2B5	IgG2b	A + C	1/16,000	1/64,000
(TiterMax^{TM})	2F1	IgG1	C		
	3A4	IgG1	A + C	1/4,000	1/16,000
	3D4	IgG1	C		
2	6A2	IgG2b	C		
(Freund)	6A11	IgG1	C		
	6C2	IgG1	C		
	9E11	IgM	C		
	10B4	IgG1	C		
	10C1	IgG1	C		

A: immunoglobulins purified from ascitic fluids. C: supernatant of cultured cells. MAbs 2B5 and 3A4 the which titer was evaluated in ELISA, were used at an initial concentration of 0.1 mg ml^{-1}. The direct and indirect protocols are respectively the protocols 2 and 1 described in Table 1.

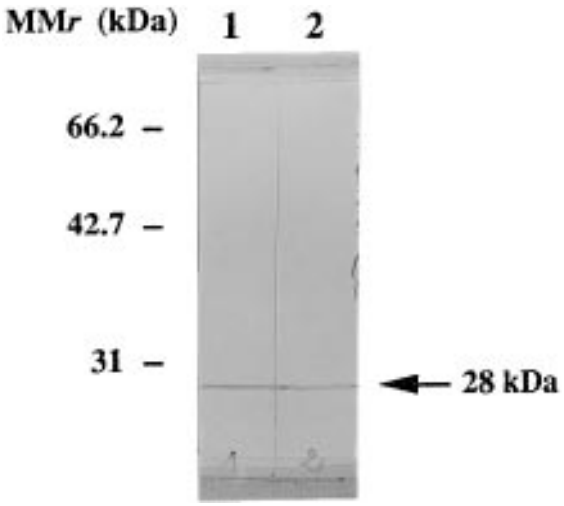

Figure 1. Immunoblotting of coat protein of GFkV with polyclonal antibodies (lane 1) and MAb 2B5 (lane 2).

IgGs on protein-A columns was 5-6 mg per ml ascitic fluid. The titer of the two MAbs was measured by ELISA and MAb 2B5 proved to have a better affinity for the virus (Table 1).

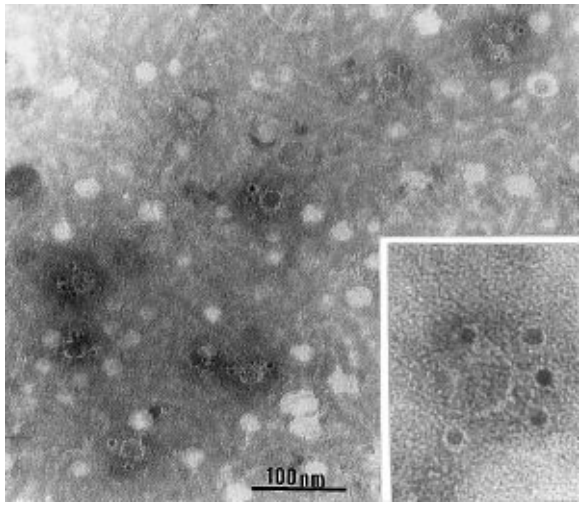

Figure 2. Immunogold labelling of a purified GFkV preparation decorated with MAb 2B5. Virus was purified from rootlets of *Vitis vinifera* Muscat d'Alexandrie.

Properties of monoclonal antibodies

Of the ten selected MAbs, seven were of IgG1 isotype, two were IgG2b and one was IgM (Table 1). All the MAbs reacted positively in ELISA with purified GFkV particles (data not shown). None of them reacted in ELISA with grapevines infected by *Nepoviruses* (ArMV, GFLV, SLRSV, RRV), *Closteroviruses* (GLRaV-1 and -3) or *Vitivirus* GVA.

In Western blot analysis the ten MAbs were able to detect a polypeptide having the same molecular weight the coat protein of GFkV (28 kDa). The reac-

Table 2. ELISA detection of GFkV with MAbs in a panel of fifty infected grapevines

MAb	2B5	2F1	3A4	3D4	6A2	6A11	6C2	9E11	10B4	10C1
A										
Number of GFkV	50	50	50	50	50	21	19	5	50	50

Sample	Polyclonal IgG	Monoclonal antibody 6A2	6C2	6A11	9E11
B					
GFkVn°3	0.234	0.481	0.084	0.050	0.039
GFkVn°17	1.213	1.831	0.327	0.475	0.228
Healthy control	0.112	0.091	0.073	0.047	0.036
Buffer	0.094	0.075	0.049	0.056	0.035

Sample	Polyclonal IgG	Monoclonal antibody 6A2	6C2	6A11	9E11
C					
GFkVn°3	nt	1.807	0.274	0.296	0.254
GFkVn°17	nt	2.254	1.325	1.156	0.985
Healthy control	nt	0.210	0.061	0.043	0.130
Buffer	nt	0.230	0.032	0.027	0.116

A: number of isolates detected by each MAb. B and C: readings of MAb 6A2 giving high responses are given in comparison with the three MAbs giving the lowest OD values (6C2, 6A11, 9E11). The two plants tested are representative of the strongly (GFkV n°17) and weakly (GFkVn°3) reacting plants from the collection.
Samples were ground in the extraction buffer with (C) or without (B) addition of 5 mM β-mercaptoethanol. All these tests were repeated twice and the OD values are the mean values after 2h incubation of substrate. nt = not tested.

tion of MAb 2B5 with the 28 kDa polypeptide was very specific since a single sharp band is observed (Figure 1). In the corresponding fraction purified from healthy grapevine, no components were detected when western blots were probed with polyclonal and monoclonal antibodies anti-GFkV (data not shown).

When purified fractions containing GFkV antigens were observed under the electron microscope after incubation with IgG 2B5 and anti-mouse gold conjugate, a specific labelling of the particles was observed (Figure 2). No labelling was observed in the control where MAb was omitted (data not shown).

Comparison of GFkV isolates

Fifty grapevine accessions of different geographical origins and infected by GFkV (as proved by ELISA using polyclonal antisera and by indexing) were tested with TAS-ELISA (Table 3, protocol 1) using culture supernatants of the ten MAbs.

Based on a threshold value of two fold the OD value of the healthy sample for a positive score, seven of the ten MAb supernatants (2B5, 2F1, 3A4, 3D4 6A2, 10B4 and 10C1) gave positive responses with all the GFkV isolates (Table 2A). The responses of the three remaining MAbs (6A11, 6C2, 9E11) ranged from clearly positive to negative depending on the isolate.

To check if those variations were due to serological variability within GFkV isolates and not only to a cumulative effect of low virus titer of the sample and low antibody titer of the MAb supernatant, a second set of tests were made including β-mercaptoethanol in the extraction buffer. The reducing properties of β-mercaptoethanol are known to increase the sensitivity of ELISA when grapevine extracts are used. With this extraction buffer, all the isolates reacted positively with all the MAbs (Table 2B, 2C).

Optimal conditions for routine ELISA detection of GFkV using MAbs

As MAb 2B5 had a better affinity for GFkV than MAb 3A4 (Table 1) and a higher OD value in ELISA at a given dilution, this MAb was further used in the

Table 3. Comparison of five ELISA protocols using either MAb 2B5 or polyclonal antibodies for the detection of GFkV in different grapevine tissues

		Protocol 1 PAb Ag MAb GaMPal S	Protocol 2 PAb Ag MAbB SavPal S	Protocol 3 MAb Ag MAbB SavPal S	Protocol 4 Prot A MAb Ag MAbB SavPal S	Protocol 5 PAb Ag PAbB SavPal S
Leaves	Infected	2.150	0.840	0.420	0.105	0.945
	Healthy	0.035	0.009	0.020	0.015	0.062
Rootlets	Infected	1.694	0.589	0.218	0.132	0.877
	Healthy	0.025	0.010	0.020	0.021	0.041
Cortical	Infected	1.572	0.410	0.093	0.025	0.614
Scrapings	Healthy	0.010	0.008	0.020	0.011	0.045

Readings of optical density after 1 h.
PAb = polyclonal antibodies; MAb = monoclonal antibody; GaMPal = goat anti-mouse alkaline phosphatase conjugate; B = biotin; Prot A = protein A; SavPal = streptavidin alkaline phosphatase conjugate; S = substrate (p-nitrophenylphosphate); Ag = antigen. Incubation conditions are detailed in text.

five ELISA procedures shown in Table 3 to set up serological diagnosis of GFkV infection.

Grapevine leaves, cortical scrapings and rootlets were all good sources for detection of GFkV using MAb 2B5 (Table 3). The woody material usually gave the lowest responses but the virus could be easily detected using protocols 1, 2 and 5. Comparison of protocols 3 and 4 showed that pre-coating of ELISA plates with protein-A did not improve detection.

ELISA protocols 1 and 2 gave clear-cut results with a large panel of plants. One hour incubation with substrate was sufficient for a clear response in all cases. In comparison with protocol 5, protocol 1 gave consistently better results, whereas protocol 2 gave responses similar to that obtained with protocol 5.

Immunoaffinity purification of GFkV

Essentially all the GFkV antigen was retained on the column after two passages of the virus sample. The elution step produced a single peak containing the antigenic material as confirmed by ELISA. The total protein concentration as measured by the Bradford method was nearly two fold higher when the virus was immunopurified from leaves (500 μg from 200 g leaves) than in the case of classical purification procedure from rootlets (700 μg obtained from 500 g rootlets).

Electron microscopy observation of samples of the elution peak revealed particles with a negatively stained center. The spectrophotometric scan of the eluted fraction exhibited a maximum near 280 nm, revealing lack of nucleic acid. Nucleic acid was not obtained after phenolic extraction of the eluted fraction (not shown).

Discussion

Difficulty to obtain sufficient quantities of highly purified virus can often be a limitation for immunization procedures, particularly when virus is phloem restricted and non-transmissible to herbaceous host plants. In the case of GFkV, the use of TiterMax^{TM} proved to be advantageous. This product is known to induce high immune responses using fewer injections and lower antigen amounts than with other adjuvants (Bennett et al., 1992). Furthermore, due to the lack of bacterial compounds, the induced immune reaction is mild and transient and causes less mortality (Bennett et al., 1992). In our case, TiterMax^{TM} proved to be efficient with a viral antigen. The antibody titer of the immunized mouse was of the same level as that of a mouse immunized using Freund's adjuvant, but the amount of injected antigen was less and the immunization period was shorter. All the MAbs produced with TiterMax^{TM} gave high OD values in ELISA whereas three out of

six of the MAbs originating from Freund's adjuvant immunization gave lower responses and were less useful for most uses.

With ten of these MAbs, no serological variability could be demonstrated among 50 GFkV isolates from various geographical origins. Similar results were obtained by Boscia et al., (1995) who obtained positive ELISA responses with two MAbs on a panel of 13 different grapevines affected by fleck. These results suggested a pronounced antigenic homogeneity of GFkV, thus encouraging the use of these MAbs for extensive serological surveys. Therefore, one of our MAbs with high titer and good affinity for GFkV was tested for routine ELISA detection. It proved to be efficient for detection with different kinds of tissues (leaves, rootlets, cortical scrapings) and could be coupled with biotin for use in DAS-ELISA without loss of specificity and sensitivity. In comparison with classical ELISA using polyclonal antiserum, procedure 1 with monoclonal antibody 2B5 gave consistently higher OD values. In addition, the responses given by healthy plants were lower which may prove important, especially in summer when GFkV detection by ELISA becomes erratic (Walter and Cornuet, 1993). However, this developed protocol still needs polyclonal antiserum for plate coating. ELISA readings obtained when coating the plate with MAb 2B5 were too low for routine use. Better results may be obtained in the future by using a cocktail of MAbs for the plate coating step.

Little information is available on the genomic organization and the taxonomic affiliation of GFkV. Nucleotide sequence determination needs the synthesis and cloning of DNA complementary to the GFkV genome. Unfortunately, the extraction of sufficient quantities of viral RNA after classical purification appeared to be very difficult. This prompted us to improve the purification of GFkV by developing an affinity chromatography protocol using MAb 2B5. The first results obtained confirmed that the method is easy-to-use and that it permits to simplify and shorten the purification procedure. The yield of protein is higher and the quality of the purification is similar to the classical procedure. Moreover, the use of certain tissues (i.e., young roots) is no longer necessary. The main problem now is that nucleic acid could not be extracted from the eluted fraction. This is probably due to the type of elution buffer used. Many viruses are sensitive to high basic pH values under which conditions their nucleic acid is released. Nevertheless, the immunopurification technique is an elegant way to obtain, in few steps, higher amounts of antigens. Work is in progress to find an appropriate elution procedure that yields virus particles containing RNA.

Acknowledgements

We thank P. Cornuet for exellent technical assistance for the immunodecoration of viral particles. This research was supported by grants of INRA (Institut National de la Recherche Agronomique), the Conseil Regional d'Alsace and LNPV Colmar.

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