Toward the Production of Monoclonal Antibodies Against Prokaryotic Pathogens of Citrus

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ABSTRACT. In order to improve the diagnosis of plant diseases associated with fastidious phloem-limited procaryotes such as mycoplasmalike organisms (MLO) and bacterialike organisms (BLO), the production of antibodies against those organisms has been envisaged. The use of monoclonal antibody technology should permit one, after immunization of mice with extracts from infected plants, to obtain hybridomas producing pathogen-specific antibodies. A model system (periwinkles infected with *Spiroplasma citri*) has been utilized to determine the best immunization procedure to be used and to develop an assay for the screening of hybridomas producing antibodies against the pathogen from those producing antibodies against the plant. The results obtained in the case of apple proliferation disease (MLO) and citrus greening disease (BLO) are presented.

For many years, the diagnosis of greening disease has been based on symptomatology followed by detection of the greening organism (GO) in the sieve tubes of the symptomatic plants by electron microscopy. The need for a quicker and more accurate method is obvious, and we have therefore envisaged the preparation of specific antibodies against the GO. This organism has not been cultured yet and is difficult to purify. It is thus available only in infected plants. In 1983, the GO was transmitted from citrus to periwinkle, a good host that multiplies GO to a higher titer than citrus (1). The development of monoclonal antibody techniques (2) also allows the production of antibodies against one antigenic determinant or epitope and should permit one to obtain antibodies specific for the GO after immunization with crude infected plant material.

The difficulty is to select the hybridomas producing antibodies only against the GO from the large number of hybridomas producing antibodies against plant proteins.

In order to find an assay for screening hybridomas we used a model system composed of *S. citri*-infected periwinkles (*S. citri*, as GO, is restricted to the sieve tubes) and monoclonal and polyclonal antibodies against *S. citri*. These antibodies were prepared with *S. citri* from culture. This model system has also been used to control the immunization efficiency.

The results obtained in different experiments conducted with greening-infected periwinkles are presented and discussed.

MATERIALS AND METHODS

Immunization of the mice

Regular immunization. Four-week-old female BALBC mice were used for immunization. On day 0 they were injected intraperitonally with 125 µl of antigen at 100 µg/ml in the presence of 125 µl of complete Freund's adjuvant.

On days 15 and 30, injections were repeated except that incomplete Freund's adjuvant was used.

On day 70, a booster injection was given intravenously with 400 µg of antigen.

Passive immunization. This immunization was done similarly to the regular one except that the antigen was first incubated for 30 min at 37 C with antibodies against proteins from healthy plants.

The antigens used for both types of immunization were either homogenates from infected midribs, infected vascular bundles or sieve tubes prepared by enzymatic digestions according to Lee and Davis (3).

Hybridoma preparation

Six x 10^7 myeloma cells X63653 Ag8 were fused with 1 x 10^8 of spleen cells in the presence of Kodak PEG 1450 according to Lane (4).

Screening assays

ELISA assay. ELISA plates are coated with the antigenic solution at 10 g/ml in carbonate buffer pH 6.9 for one night at 4 C, washed three times with PBS pH 7.2 +2% Tween 20 and then saturated with 4% bovine serum albumin (BSA) for 30 min at 37 C.

After washing, the hybridoma supernatants (or other antisera) were added and the plates were incubated for 2 hr at 37 C followed by one night at 4 C. After washing, sheep anti-mouse IgG F(ab)'2 fragments labeled with peroxidase (diluted 1/1000) were added for 30 min at 37 C. Bound, labeled antibody was revealed with 2,2' azino di 3 ethyl benzothiozoline sulphonate (ABTS).

The optical density (OD) of the color reaction was recorded at 405 nm after 30 min.

Biotin-Avidin ELISA. Coating of the plates was done as described above except that 20 μg/ml of antigen were used. All the washes were done with PBS + 0.01% gelatin.

Plates were saturated with 0.5% gelatin for 30 min at 37 C. The hybridoma supernatants were incubated as in the previous assay, but detected with sheep antimouse IgG F(ab)'2 fragments labeled with biotin (1/2000 for 1 hr at 37 C). Avidin peroxidase (Sigma) diluted 400-fold was added and incubated for 20 min at room temperature. The substrate was ABTS as previously described.

Both ELISA assays were differential assays in which each hybridoma supernatant was tested against extracts from both infected and healthy plants. A hybridoma giving a positive ELISA with both extracts was assumed positive against plant proteins while a hybridoma giving a positive only with infected extracts was one producing antibodies against GO. An ELISA was considered positive when the optical density was above 0.1.

Immunofluorescence. Thirty three μm longitudinal sections of healthy and infected plant midribs were cut with a freezing microtome. The sections were incubated with the hybridoma supernatant for 1 hr at room temperature and then with anti-mouse sheep IgG labeled with fluorescein isothiocyanate (FITC) diluted 10,000-fold in the same dilution of Evans blue for 1 hr. After washing with PBS-Tween they were observed with a Zeiss III RS fluorescent microscope with a filter combination BP 455-490/FT 510/LP520.

RESULTS

Evaluation of ELISA and biotin-avidin ELISA assays for the screening of hybridomas. Evaluation of the assays was done using extracts from *S. citri*-infected periwinkles and monoclonal antibodies against the major protein of the membrane of *S. citri*. An antiserum produced in a rabbit by injection of the cultured organism (polyclonal antibody) was also used.

The results obtained for polyclonal or monoclonal antibodies tested against *S. citri* from culture or *S. citri*-infected periwinkles midribs are shown in table 1.

The polyclonal and monoclonal antisera gave a positive ELISA when tested against *S. citri* from culture, but the OD was more than 10 times higher with the polyclonal serum. Only the polyclonal antiserum gave a positive reaction with infected periwinkle midribs and the OD was rather low (0.143). The relative amount of *S. citri* proteins versus plant proteins in infected midribs was apparently too low to be detected with monoclonal antibodies. For this reason, infected vascular bundles were prepared (see Materials & Methods) which contained both xylem and phloem elements, with most of the parenchyma cells removed.

TABLE 1
DIFFERENTIAL ELISA WITH POLYCLONAL AND MONOCLONAL ANTISERA PREPARED FROM CULTURED *SPIROPLASMA CITRI* CELLS

Antigens	Anti-*S. citri* polyclonal antiserum	Anti-Spiralin monoclonal antiserum
Healthy midribs	0.050[z]	0.014
Infected midribs	0.143	0.020
Healthy bundles	0.060	0.010
Infected bundles	0.655	0.020
S. citri cells	> 2.0	0.200

[z]Optical density at 405 nm.

When such infected bundles were used as the antigen source, the OD obtained with the polyclonal serum was about five-fold higher than with the infected midribs, but the assay was still negative with monoclonal antibodies.

Since better purification of the antigen was difficult, we tried to increase the sensitivity of the ELISA assay itself by adding a biotin-avidin step.

The results of this assay are shown in table 2. The optical density obtained when the monoclonal antiserum was tested against the *S.citri* culture indicates that the biotin-avidin step results in about a six-fold increase in assay sensitivity (OD 1.24 vs 0.2). This increase is sufficient to obtain a positive ELISA when infected midribs or bundles are used as antigens (0.120 and 0.224 respectively).

In conclusion, by using enzyme-purified infected bundles as antigen and a biotin-avidin ELISA, the assay was sensitive enough to detect *S. citri* antigens present in low amounts in a mixture of plant antigens with a monoclonal serum. This assay will be used to screen the hybridomas produced after immunization of mice with greening-infected plant extract.

Evaluation of two immunization protocols. Two different immunization protocols were used to obtain antibodies against *S. citri* after injection of *S. citri*-infected periwinkle extracts. As described in the Materials & Methods infected vascular bundles were used as antigens for immunization in the presence or absence of antibodies against plant proteins. The screening assay was the biotin-avidin ELISA. The results (table 3) show that 1% *S. citri*-specific hybridomas are obtained with a regular immunization (14 of 1452) whereas around 2.5% are obtained with a passive immunization (12 of 440).

In conclusion, the amount of *S. citri* antigens in infected bundles is high enough to induce an antibody response in the mouse. The neutralization of plant antigens with the anti-plant serum results, as expected, in an increase of the number of *S. citri*-specific hybridomas. Those antibodies specific for *S. citri* in the ELISA test have been used to detect *S. citri* cells directly in sections from infected periwinkles by immunofluorescence. A specific fluorescence was observed in the sieve tubes of infected, but not in sieve tubes of healthy periwinkles. Therefore, immunofluorescence can be used as an additional hybridoma screening test.

TABLE 2
ELISA AND AVIDIN-BIOTIN ELISA WITH ANTI-SPIRALIN MONOCLONAL ANTISERUM

ELISA technique	ANTIGENS				
	Healthy midribs	*S. citri*-infected midribs	Healthy bundles	*S. citri*-infected bundles	*S. citri* cells
ELISA	0.014[z]	0.020	0.010	0.020	0.200
Avidin-biotin ELISA	0.086	0.120	0.080	0.224	1.240

[z]Optical density at 405 nm.

TABLE 3
IMMUNIZATION OF MICE WITH *SPIROPLASMA CITRI*-INFECTED BUNDLES

Type of immunization	Total number of clones	Number of *S. citri* specific clones
Regular immunization	1,452	14
Passive immunization	440	12

Trials to obtain GO specific clones. Four different fusion experiments were performed to obtain antibodies against the GO. The results are shown in table 4. In the first experiment, immunization of the mouse was done with infected midribs and 67 hybridomas were obtained, two of which gave a positive reaction only with infected midribs. After cloning, these two hybridomas stopped producing antibodies. In the second and third experiments, infected bundles were used as antigens for immunization. In the third experiment, they were first mixed with a serum prepared against healthy bundles. Of 540 and 1,251 hybridomas obtained, two and five, respectively, were specific for infected bundles. However, these hybridomas also gave a positive reaction when tested against bundles from periwinkles infected with mollicutes such as the MLOs of phyllody and apple proliferation diseases or *S. citri*. No positive ELISA was obtained with healthy bundles.

In a fourth experiment a more purified antigen, referred to as purified sieve tubes, was used. Most of the parenchyma and xylem elements had been eliminated from this preparation. Of 1,087 hybridomas obtained, 33 were specific for infected bundles. Those hybridomas can be divided into two groups according to the value of the OD obtained in the screening ELISA assay. Nineteen give an OD lower or equal to 0.2, similar to that obtained with the *S. citri* model, and 14 gave an OD between 0.3 and 0.8. The characterization of these clones is underway.

DISCUSSION

By using a model system and hybridoma technology we have shown that it was possible to obtain monoclonal antibodies against a phloem-limited procaryote by immunization of mice with extracts from infected periwinkle plants.

A screening assay has been developed to select the procaryote-specific hybridomas from those producing antibodies against plant material. So far this technique has not permitted the development of GO-specific antibodies from greening-infected periwinkles. Different reasons can be advanced to explain this failure. In

TABLE 4
IMMUNIZATION OF MICE WITH VARIOUS GREENING-INFECTED TISSUES AND EVALUATION OF HYBRIDOMAS

Exp. No.	Immunization with greening-infected tissue	Number of hybridomas	Number of hybridomas giving a positive ELISA for greening-infected bundles only	Remarks
1	Midribs	67	2	Lost activity after cloning
2	Bundles	548	2	Positive with bundles infected with other diseases
3	Bundles + serum against healthy bundles	1.251	5	
4	Sieve tubes	1.087	33	19 gave an OD of 0.2 14 gave an OD of 0.3

the first experiment immunization was done with a relatively crude plant extract (midribs) because the conditions of immunization we used with *S. citri* had not yet determined. When using infected bundles, as in the case of *S. citri*, we obtained some hybridomas specific for infected material. Unfortunately, they also gave a positive ELISA with periwinkles infected with other diseases, showing that they were specific to a protein induced in the plant by a phloem-limited procaryote, but were not specific of the GO itself. Such hybridomas were not detected in the case of *S. citri* immunization because the screening assay was done against *S. citri* cells and not against *S. citri*-infected bundles.

In the last experiment purified sieve tubes were used. It is still too early to know if GO-specific hybridomas are present among the 330 giving a positive ELISA with infected material. If those clones turn out to be specific for proteins induced by infection only, they will be used in association with a serum prepared against healthy sieve tubes to further adsorb the antigen before immunization.

NOTE ADDED IN PROOF

We have now obtained two hybridomas specific for the Indian strain of the GO.
Garnier et al. (1987) Annals Microbiol. Inst. Past. 138 (in press)

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