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Authors

Gao, S.
Garnier, M.
Bové, J. M.

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Production of Monoclonal Antibodies Recognizing Most Asian Strains of the Greening BLO by *In Vitro* Immunization With an Antigenic Protein Purified From the BLO

Shoujiang Gao, Monique Garnier, and Joseph M Bové

ABSTRACT. Since 1987, we have produced ten different monoclonal antibodies (MA) specific for the greening BLO. Two were produced against an Indian (Poona) strain, five against a strain from China (Fujian), and three against a strain from South Africa (Nelspruit). The use of these various MAs for the detection of the greening BLO has shown that each MA is very specific for the strain used to immunize the mice and, therefore, only this homologous strain could be detected. We have now purified an antigenic protein of the Poona strain of the BLO, by immunoaffinity chromatography using a MA against this protein. The protein, only available in very low amounts, has been used to produce MAs by *in vitro* immunization of spleen cells. Three MAs have been obtained. One of these recognizes all the Asian strains tested except the Chinese strain. The two others recognize most of the Asian strains. None of them recognized the African strain.

Study of the greening bacterium-like organism (BLO) has been difficult since the organism has not been cultured *in vitro*, and detection of the BLO could be done only by electron microscopy.

The first reagents specific for the greening BLO were produced in 1987, and consisted of two monoclonal antibodies (MA) prepared against a strain from Poona (India) (4). It was soon shown that these MAs were very specific for the Poona strain and did not react with strains from other geographical origins. Eight other MAs produced against strains from China and South Africa also turned out to be highly strain specific (3) and, consequently, these MAs could not be used for diagnosis of greening disease.

All the above MAs were produced by using as immunogen, homogenates of phloem tissue from BLO-infected periwinkle plants. In this relatively crude preparation, BLO antigens were present in low amounts. This probably explains why MAs against strain-specific epitopes were obtained, such epitopes being more antigenic than epitopes common to several strains (5).

In order to produce antibodies recognizing most or all strains of the BLO, we have now purified by immunoaffinity chromatography the antigenic pro-

tein of the greening BLO (Poona strain) recognized by MA 10A6. By *in vitro* immunization of splenocytes with this protein, new MAs recognizing most strains of the BLO have been obtained. A serological classification of the BLO is presented here.

MATERIALS AND METHODS

Plant material. Healthy and Poona BLO-infected periwinkle plants were grown in a greenhouse at 28 C during the day and 23 C at night.

Purification of the antigenic protein. Three hundred grams of periwinkle midribs were ground in a Waring blender in the presence of 1.2 liters of phosphate buffer saline (PBS) 500 (PBS containing NaCl 500mM). The homogenate was filtered through four layers of cheese cloth, centrifuged at $1,200 \times g$ for 10 min and the resulting supernatant heated at 100 C for 5 min and centrifuged at $15,000 \times g$ for 20 min. The supernatant proteins were precipitated with 65% ammonium sulfate for 30 min and centrifuged for 30 min at $15,000 \times g$. The resulting pellet was resuspended in PBS 500 (= C65). The supernatant was treated with 10% trichloroacetic acid (TCA) and left precipitated for 20 min after which it was centrifuged at $15,000 \times g$ for 30 min

The pellet was resuspended in PBS 500, adjusted to pH 7.4 and mixed with the first resuspended pellet (C65). One volume of chloroform was added to the resuspended pellets and vortexed. The phases were separated by centrifugation at $2,000 \times g$ for 10 min, the aqueous phase collected and concentrated in a dialysis bag against 10% polyethylene glycol (PEG) 6000 for 1 hr at 4 C. After centrifugation for 30 min at $15,000 \times g$, the pellet was resuspended in TD buffer (Tris-HCl pH 7.4; 5 mM EDTA, 0.0176% ascorbic acid, 100 mM PMSF). The resulting preparation was incubated with 0.5% Triton X-100 and 0.5% desoxycholate for 1 hr at room temperature and centrifuged for 1 hr at $180,000 \times g$. The solubilized antigenic protein was purified from the resulting supernatant by immunoaffinity chromatography with MA 10A6 (4, 7). A volume of 25.5 ml of antigenic solution was put on the column, the column was washed with PBS containing 0.5% Triton X-100 and 0.5% desoxycholate, then with PBS 500 alone. Elution of the antigen was done with a total of 15 ml of 0.1 M diethyl amide pH 11. All fractions obtained during the purification procedure were tested by DAS-ELISA (2, 4) for the presence of antigen reacting with MA 10A6. The fraction loaded on the immunoaffinity chromatography column as well as the eluted fractions were also analysed by polyacrylamide gel electrophoresis and western blotting (6).

Production of MAs by *in vitro* immunization. We used the technique of Borrebaeck (1). The Balb/C mouse

splenocytes were incubated with 300 μ l of antigenic solution prepared by concentrating the 15 ml of eluate from immunoaffinity chromatography with a centricon 10 (Amicon) and sterilized by filtration through a 0.22 μ m filter. The incubation was for 120 hr at 37 C. Fusion of the *in vitro* immunized splenocytes with myeloma cells was done as described (4).

RESULTS

Purification of the antigenic protein recognized by MA 10A6. Table 1 shows the results of the immunoaffinity chromatography purification of the antigen recognized by MA 10A6. The fraction incubated with the immunoaffinity column and called CHLAconS had a volume of 25.5 ml and contained 8.951 mg of proteins per ml. The OD at 405 nm of the ELISA assay on this fraction was 3.76 per μ g of protein. The run-through fraction of the column (L1) had the same volume and gave negative results by ELISA, showing that all the antigenic protein was retained on the column. Two successive washings of the column with 25 ml (L2) and 50 ml (L3) of buffer respectively gave, as expected, negative ELISA results. Elution of the attached antigen was done with six times 5 ml fractions of diethyl amide. ELISA results showed that most of the antigen was eluted in the first 15 ml.

The proteins present in 2 ml of the fraction CHLAconS and in the pooled eluted fractions (E1-E4) were analysed by PAGE and western blot. Fig. 1 shows

TABLE 1
PURIFICATION OF THE ANTIGENIC PROTEIN RECOGNIZED BY MA 10A6
BY IMMUNOAFFINITY CHROMATOGRAPHY

Fraction	CHLAconS ^a	L1	L2	L3	E1	E2	E3	E4	E5	E6	E
Volume (ml)	25.5	25.5	25	50	5	5	5	5	5	5	30
Protein (mg)	8.951	—	—	—	—	—	—	—	—	—	—
DAS-ELISA (OD)	33,660	0	0	0	17,600	9,882	2,767	732	267	367	31,617
OD/ μ g	3.76	—	—	—	—	—	—	—	—	—	—
Percentage OD (%)	100	0	0	0	52.29	29.36	8.22	2.18	0.79	1.1	93.932

^aCHLAconS = 10A6 antigenic preparation before immunoaffinity chromatography; L1 = run through fraction; L2 and L3 = washing fractions; E1 to E6 = Different elution fractions; E = mixture of all eluted fractions.

Fig. 1. PAGE analysis (silver stained) of proteins in the fraction CHLAconS and in the pooled eluted fractions.

Lane 1: eluted fractions from healthy periwinkles; Lane 2: eluted fractions from GO (Indian strain) infected periwinkles; Lane 3: fraction CHLAconS from healthy periwinkles; Lane 4: fraction CHLAconS from GO-infected periwinkles; M: molecular weight markers (Kd).

that many proteins are present in periwinkle extracts prepared from both healthy (track 3) or BLO-infected (track 4) periwinkle midribs before affinity chromatography. However no protein bands could be seen in the eluate (E1-E4) from infected-periwinkle (track 2), whether the gels were stained with Coomassie blue or silver nitrate.

Even though no electrophoretic bands could be seen in the case of the eluates on the gel, a gel on which 200 μ l of the fractions were run was electrotransferred onto a hybond C-extra

membrane (western blotting). The transferred antigens were revealed with MA 10A6. Results are shown in Fig. 2. Two bands of 20 and 22 kD were strongly labeled, and a band of 42 kD was faintly visible in the case of the eluate from infected extracts (track 2). The same bands were also present but in very low amounts in the fraction CHLAconS loaded on the affinity column (track 4). As expected, no bands were present in similar fractions obtained from healthy periwinkle midribs (tracks 1 and 3). The fact that three bands were stained with the same antibody (10A6) shows that the antigenic protein of 42 kD is composed of two serologically related subunits of 20 and 22 kD.

Fig. 2. Western-blot of gel in Fig. 1 analyzed with MA 10A6.

Lane 1: eluted fractions from healthy periwinkles; Lane 2: eluted fractions from GO (Indian strain) infected periwinkles; Lane 3: fraction CHLAconS from healthy periwinkles; Lane 4: fraction CHLAconS from GO-infected periwinkles; M: molecular weight markers (Kd).

Production of MAs with the purified antigenic protein. MAs were produced against the purified protein by *in vitro* immunization. With this technique, only a few ng of antigens are required for immunization. Three stable hybridomas 2C11, 2B6, and 1A5 were obtained. The MAs 2C11 and 2B6 were IgM while 1A5 was an IgG 1. The specificity of the MAs produced by the various hybridomas was tested by IF and/or DAS-ELISA with plant material infected with BLO of different geographical origins. Table 2 shows that MA from hybridoma 1A5 recognized all Asian strains tested except the two Chinese strains. In addition to the Chinese strains, AM 2C11 did not react with the strain from Malaysia and AM 2B6 with the strains from Taiwan and Malaysia. None of the MAs reacted with the African strain (Nelspruit) tested. The use of the various MAs pro-

duced against the greening BLO, has allowed us to define 7 different serogroups as seen in Table 3.

CONCLUSIONS

In order to produce a serological reagent able to recognize most if not all strains of the greening BLO, an antigenic protein of the Poona strain of the BLO has been purified from BLO-infected periwinkle midribs. Using the purified protein, we were able to produce three MAs by *in vitro* immunization of spleen cells. One of the MAs is able to recognize all the strains tested except the two Chinese strains and the African strain. This is in agreement with the results obtained with DNA probes which have shown that Asian and African BLOs are probably two different species or subspecies (8, 9). For the first time, a serological reagent

TABLE 2
DETECTION OF THE GREENING BLO BY IMMUNOFLUORESCENCE (IF) OR DAS-ELISA WITH MONOCLONAL ANTIBODIES 2C11, 2B6 AND 1A5

BLO STRAIN	2C11	2B6	1A5	
	IF	IF	IF	DAS-ELISA
India (Poona)				
Periwinkle	+ ^a	+	+	>2 ^c
Citrus	+	+	+	>2
Indonesia (Bali)				
Citrus	+	+	+	>2
Philippines (Lipa city)				
Citrus (1970) (strain 1)	+	+	+	>2
Citrus (1989) (strain 2)	+	+	+	>2
Periwinkle (1989) (strain 3)	+	+	+	>2
Thailand (Bangkok)				
Periwinkle	+	+	+	>2
Citrus	+	+	+	>2
China (Fuzhou)				
Periwinkle (strain 1)	— ^b	—	—	0.003
Citrus (strain 2)	—	—	—	0.002
Taiwan (Taipei)				
Citrus	+	—	+	0.145
Malaysia (Sarawak)				
Citrus	—	—	+	>2
South Africa (Nelspruit)				
Periwinkle	—	—	—	0.000
Citrus	—	—	—	0.007

^aFluorescence in the phloem.

^bNo fluorescence in the phloem.

^cOD at 405 nm.

TABLE 3
SEROLOGICAL CLASSIFICATION OF THE VARIOUS GREENING BLO STRAINS

STRAINS	HYBRIDOMAS													SEROGROUP
	2D12	10A6	2B6	2C11	1A5	10F4	5H10	6G1	11H6	12E12	MG8	10H8	14A1	
Indonesia (Bali)	+	+	+	+	+	-	-	-	-	-	-	-	-	I
Indonesia (Bali)	-	+	+	+	+	-	-	-	-	-	-	-	-	II
Philippines (Lipa city) (1970, strain 1)	-	+	+	+	+	-	-	-	-	-	-	-	-	II
Philippines (Lipa city)	-	+	+	+	+	-	-	-	-	-	-	-	-	II
Philippines (Lipa city)	-	+	+	+	+	-	-	-	-	-	-	-	-	II
Thailand (Bangkok)	-	-	+	+	+	-	-	-	-	-	-	-	-	III
Philippines (Lipa city)	-	-	+	+	+	-	-	-	-	-	-	-	-	III
Taiwan (Taipei)	-	-	-	+	+	-	-	-	-	-	-	-	-	IV
Malaysia (Sarawak)	-	-	-	-	+	-	-	-	-	-	-	-	-	V
China (Fuzhou, strain 1)	-	-	-	-	-	+	+	+	+	+	-	-	-	VI
China (Fuzhou, strain 2)	-	-	-	-	-	+	+	+	+	+	-	-	-	VI
South Africa (Nelspruit)	-	-	-	-	-	-	-	-	-	-	+	+	+	VII

reacting with many BLO strains is available. Its use in combination with MAs specific for the BLOs from China and South Africa may provide a recon-

stituted polyclonal antibody for the detection of all BLO strains. This needs further investigation.

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