Production of Monoclonal Antibodies Against *Spiroplasma citri*

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ABSTRACT

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From four independent fusions, 13 stable hybridoma cell lines secreting specific monoclonal antibodies against distinct epitopes on *Spiroplasma citri* 'Maroc' were selected from hybridomas produced by fusing NS-1/1-Ag4-1 murine myeloma cells with splenic cells from mice immunized with *Spiroplasma citri* (Maroc). With indirect ELISA with biotinylated anti-mouse Ig, the antibodies secreted by 9 of these 13 clones reacted specifically with strains Maroc, C189, CB-1, Arizona, Aceratagallia, SCC, Scaph, L163, B106, and BR-3, but not with strains Algeria, ASP-1, Israel, and Iran of *S. citri*. The monoclonal antibodies also distinguished *S. citri* from corn stunt spiroplasma (strains I-747 and Miss E), honeybee spiroplasma (AS 576), 277F spiroplasma (277F), green leaf bug spiroplasma (LB-12), Maryland flower spiroplasma (M55), Cocos spiroplasma (N525), P40 spiroplasma (P40) of the same serogroup (group I), and from the other 19 spiroplasma strains belonging in the 18 other serogroups. Isotyping of the monoclonal antibodies was carried out by using the Ouchterlony double-diffusion method with class- and subclass-specific antibodies for mouse IgG1, IgG2a, IgG2b, IgG3, and IgM. Three clones were of the IgM isotype, four of the IgG1 isotype, and six of the IgG3 isotype. These specific monoclonal antibodies are useful for differentiating the strains of *S. citri* from different geographic areas.

Additional key words: hybridoma technique, spiroplasma serogroup, spiroplasma strain.

Serological methods are important for identifying spiroplasmas (17). Until now, only conventional polyclonal antisera or antiascites have been used in serological methods, such as the deformation test (6,18,23) and the growth inhibition test (10) for serotyping spiroplasmas (3,11,12,20–22). Common antigenic determinants among spiroplasma strains often make serological comparisons of the same species or within the serogroup difficult to interpret (12,13,19–21,23).

In 1975, Kohler and Milstein (15) showed that somatic hybridization of lymphocytes and myeloma cells could be used to establish continuous culture of specific antibody-producing cells. The antibodies produced in such culture are fundamentally different from those in antisera or antiascites obtained by conventional means, because virtually unlimited amounts of homogeneous monoclonal antibodies are produced against desired antigens or haptens (24).

The purpose of the research reported here was to use the hybridoma serological technique to obtain more highly specific antisera than can be obtained with polyclonal antibodies produced by conventional methods. We were particularly interested in testing whether the technique would permit differentiation of *Spiroplasma citri* from spiroplasmas in group I and from strains of *S. citri* from different geographic areas.

MATERIALS AND METHODS

Antigen preparation. A log-phase culture of *Spiroplasma citri* (Maroc strain, ATCC 27556), cultivated in 500 ml of R-2 broth medium (5) was harvested by centrifugation at 20,000 *g* for 30 min. Pellets were washed twice and then resuspended in 5 ml of phosphate-buffered saline (PBS) (pH 7.4) for further dilution.

Monoclonal antibody production. Six-week-old female BALB/c mice were immunized. For the first immunization 0.1 ml of *S. citri* (Maroc) antigen preparation diluted in PBS (300 μg protein, Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, CA) was mixed with 0.1 ml of Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY) and injected intraperitoneally. The second immunization on day 21 was similar to the first except that Freund's incomplete adjuvant was used. One week after the second injection, the same amount of *S. citri* preparation without adjuvant was injected intravenously. The mice were sacrificed 3 days later and their spleens were removed for cell fusions.

The murine myeloma cell line P3-NS-1/1-Ag4-1 (NS-1) used in the fusion was grown in RPMI-1640 medium supplemented with 15% fetal bovine serum (complete medium) (Gibco Laboratories). All myeloma cells were cultured in a humid atmosphere containing 7% CO_2 at 37 C. Myeloma cells were detached from the wall of the culture flask, and the suspension was diluted twofold with fresh medium 1 day before the fusion.

Spleen cells (10^8), released from intact spleens by repeatedly injecting the spleen with serum-free RPMI-1640 medium, were then mixed with the myeloma cells (5×10^7) and centrifuged at 200 *g* for 10 min. The pellet was gently loosened and resuspended by dropwise addition of 1.5 ml of 40% (w/v) PEG 4000 (Sigma Chemical Co., St. Louis, MO) in serum-free RPMI-1640 medium over a period of 45 sec. The PEG suspension was first diluted by dropwise addition of 5 ml of serum-free RPMI-1640 medium over a period of 10 min, then an additional 10 ml of the same medium was similarly added over another 10 min. The cell mixture was centrifuged at 200 *g* for 10 min and the pellet was resuspended in 80 ml of RPMI-1640 medium supplemented with 15% fetal bovine serum (complete medium). After 3 hr of incubation in a CO_2 incubator, the cell suspension was diluted by an equal volume of complete medium supplemented with 2× concentrated HAT (2×10^{-4} M hypoxanthine, 8×10^{-7} M aminopterin, and 3.2×10^{-5} M thymidine) (Sigma Chemical Co.). This medium allowed only the spleen-myeloma hybrid cells to grow. The fusion mixture was plated out into seven 96-well plates. After day 7, half of the medium was removed and replaced with fresh HAT medium every 3 days; after day 19, HT medium (complete medium supplemented with 10^{-4} M hypoxanthine, and 1.6×10^{-5} M thymidine) was used in place of the HAT medium.

The supernatant from wells containing hybridomas were tested for the presence of antibodies by indirect ELISA with biotinylated

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anti-mouse IgG and IgM (Vector Laboratories, Burlingame, CA) (16). The 96-well microtiter plates (Vangard International, Neptune, NJ) were first coated with the antigen preparation of *S. citri* diluted in 0.05 M carbonate buffer (pH 9.6) (protein content = 10 μg/ml, Bio-Rad protein assay). Then, hybridoma culture fluids (in which cells have grown up to one-sixth of the well area) was added. The biotinylated anti-mouse Ig and the avidin-biotinylated peroxidase were sequentially incorporated before the *o*-phenylenediamine substrate was added. The reaction was stopped with 3 N H_2SO_4, and the plates were read at 490 nm in an ELISA reader (Flow Laboratories, Inc., McLean, VA). Positive clones were subcultured and allowed to increase to mass cultures. For each of these clones, one part was frozen in liquid nitrogen and the other part was immediately selected for monoclones derived from single cells by limiting dilution method. Single-cell clones were selected microscopically from plates in which 35% of the wells contained monoclonal hybridomas (14) and these were cultured in complete RPMI-1640 medium for further processing and freezing.

Antibody isotype determination. Antibody class was determined by the Ouchterlony double-diffusion method by using rabbit antisera for mouse IgG1, IgG2a, IgG2b, IgG3, and IgM (Miles Laboratories, Inc., Elkhart, IN) (14). Monoclonal antibodies were precipitated from the culture supernatant with 50% saturated ammonium sulfate, concentrated, and resuspended to one-tenth of the original volume with PBS before dialysis against PBS (pH 7.4).

Ascitic fluid production. Hybridoma cells were grown in mice by injecting pristane-primed BALB/c mice with 10^6 cells. The ascitic fluid was harvested after 10–20 days by tapping the peritoneum with a 19-gauge syringe needle (16).

ELISA titer tests. Monoclonal antibodies used in ELISA titer tests were harvested from culture supernatants after cultures reached $5–7 \times 10^6$ cells per milliliter, or directly from ascitic fluid. Indirect ELISA with biotinylated anti-mouse IgG and IgM was used. The protein content of *S. citri* antigen preparation used for coating was 10 μg/ml. A 1:5 dilution series was used with a minimum dilution of 1:500 for ascitic fluid, and a 1:2 dilution series was used with a minimum dilution of 1:25 for culture supernatant.

Monoclonal antibody specificity. Indirect ELISA with biotinylated anti-mouse Ig was used in the specificity studies. Forty-one spiroplasma strains belonging in 19 different serogroups (20) were used as antigens (kindly provided by R. F. Whitcomb and R. E. Davis, USDA, Beltsville, MD; and L. R. Nault, Ohio State University, Wooster) (Table 1). Nine of the 13 monoclones with high ELISA titers were used in this study. All of the spiroplasma antigens were prepared the same way as the coating antigen of *S. citri*, but the protein content was diluted to 5 μg per milliliter. Monoclonal antibodies purified from culture supernatant, and the conventionally produced polyclonal antibodies from mouse ascitic fluid (5) were prepared as for antibody isotyping. Antibodies used in ELISA were diluted to 5 μg of protein per milliliter in PBS (pH 7.4).

RESULTS

A total of 35 hybridoma clones from four independent fusions tested positive for antibodies specific for *S. citri* (Maroc) were

TABLE 1. Spiroplasma strains used in serological comparison with monoclonal antibodies to *Spiroplasma citri*

Binomial or common name	Group or subgroup[a]	Strain	Source or ATCC no.
Spiroplasma citri	I-1	Maroc (R8A2)	27556
		C189	27665
		CB-1	R. F. Whitcomb
		Arizona	R. F. Whitcomb
		Aceratagallia	R. F. Whitcomb
		SCC	R. F. Whitcomb
		Scaph	R. F. Whitcomb
		L163	R. F. Whitcomb
		B106	R. F. Whitcomb
		BR-3	R. E. Davis
		Algeria	R. F. Whitcomb
		ASP-1	R. F. Whitcomb
		Israel	R. F. Whitcomb
		Iran	R. F. Whitcomb
Honeybee spiroplasma	I-2	AS-576	29416
Corn stunt spiroplasma	I-3	I-747	29051
		Miss E	L. R. Nault
277F spiroplasma	I-4	277F	29761
Green leafbug spiroplasma	I-5	LB 12	33469
Maryland flower spiroplasma	I-6	M55	33502
Cocos spiroplasma	I-7	N525	33287
P40 spiroplasma	I-8	P40	R. F. Whitcomb
S. floricola	III	23-6	29989
S. apis	IV	SR-3	33095
		B31	33834
S. mirum	V	SMCA	29335
Y32 spiroplasma	VI	Y32	33835
MQ-1 spiroplasma	VII	MQ-1	33825
EA-1 spiroplasma	VIII	EA-1	33826
CN-5 spiroplasma	IX	CN-5	33827
Mosquito spiroplasma	X	AES-1	35112
MQ-4 spiroplasma	XI	MQ-4	R. F. Whitcomb
DU-1 spiroplasma	XII	DU-1	R. F. Whitcomb[b]
CC-1 spiroplasma	XIII	CC-1	R. F. Whitcomb[b]
EC-1 spiroplasma	XIV	EC-1	R. F. Whitcomb[b]
I-92 spiroplasma	XV	I-92	R. F. Whitcomb[b]
CB-1 spiroplasma	XVI	CB-1	R. F. Whitcomb[b]
DF-1 spiroplasma	XVII	DF-1	R. F. Whitcomb[b]
TN-1 spiroplasma	XVIII	TN-1	R. F. Whitcomb[b]
PUP-1 spiroplasma	XIX	PUP-1	R. F. Whitcomb[b]
I-25 spiroplasma	XXI	I-25	R. F. Whitcomb[b]

[a] Serogroups according to (20) and R. F. Whitcomb (*personal communication*).
[b] (*Unpublished*).

selected. Of these clones, 13 were stable. These were maintained and used to produce monoclones that secreted antibodies specific for *S. citri* (Maroc). When these 13 monoclones were isotyped by the Ouchterlony method, three clones were of the IgM isotype, four clones were of the IgG1 isotype, and the other six clones were of the IgG3 isotype (Table 2).

TABLE 2. Isotyping and titer of *S. citri* monoclonal antibodies in culture supernatant and ascitic fluid

Cell line	Antibody isotype	ELISA titer[a]	
		Culture supernatant	Ascitic fluid
MA1	IgM	25	NT[b]
MA2	IgG1	3,200	7,812,500
MA3	IgM	50	5,000
MA4	IgG3	3,200	7,812,500
MA5	IgG3	1,600	NT
MA7	IgG3	3,200	1,562,500
MA8	IgG3	6,400	7,812,500
MA10	IgG1	200	62,500
MA15	IgG1	6,400	7,812,500
MA19	IgG1	1,600	1,562,500
MA23	IgG3	1,600	NT
MA24	IgM	25	NT
MA26	IgG3	3,200	1,562,500

[a] ELISA titer is the reciprocal of the highest dilution that gave $A_{490\ nm} > 0.1$.
[b] NT = not tested.

ELISA tests for antibody titers of cell culture supernatants ranged from 50 to 6,400, while the titers of ascitic fluids ranged from 5,000 to 7,812,500 when reacted with *S. citri* (Maroc) (Table 2). In these clones, the titers of antibodies from clones of IgM were lower than those from clones of IgG class. This may be due to the lability in culture supernatant under 37 C incubation in addition to the clone nature (such as producing antibody against minor antigenic determinant of *S. citri* antigen). The four clones with lower titer were not further used in the specificity study.

All of the nine monoclonal antibodies reacted specifically with *S. citri* strains C189, CB-1, Arizona, Aceratagallia, SCC, Scaph, L163, B106, and BR-3 isolated in the United States, and strain Maroc isolated in North Africa, but did not react with strains Algeria, ASP-1, Israel, and Iran. No reactions were detected between our monoclonal antibodies with honeybee spiroplasma (AS-576), corn stunt spiroplasma (strains I-747 and Miss E), 277F spiroplasma (277F), green leaf bug spiroplasma (LB-12), Maryland flower spiroplasma (MS5), Cocos spiroplasma (N525), P40 spiroplasma (P40) of the group I, and the other 19 spiroplasma strains belonging to the other 18 different serogroups (Table 3). The conventionally produced polyclonal antibody, however, reacted not only with all of the 14 strains of *S. citri* that were tested, but also

TABLE 3. Absorbance at 490 nm of monoclonal antibodies (Ab) to *Spiroplasma citri* with spiroplasmas in indirect enzyme-linked immunosorbent assay

Antigen[a]		Antiserum[b]									
		Ab of cell line									Polyclonal antibody[c]
Group	Strain	MA2	MA4	MA5	MA7	MA8	MA15	MA19	MA23	MA26	
I-1	Maroc	7+[d]	7+	8+	7+	7+	8+	7+	7+	7+	8+
	C189	8+	7+	7+	7+	7+	9+	7+	7+	7+	8+
	CB-1	6+	7+	6+	7+	6+	6+	6+	6+	7+	6+
	Arizona	7+	7+	7+	7+	7+	7+	7+	7+	7+	7+
	Aceratagallia	7+	7+	7+	6+	7+	7+	6+	7+	7+	6+
	SCC	7+	7+	7+	6+	7+	6+	7+	7+	7+	6+
	Scaph	6+	7+	7+	7+	7+	7+	6+	6+	7+	7+
	L163	7+	7+	7+	6+	6+	7+	7+	7+	6+	7+
	B106	8+	7+	8+	7+	7+	8+	7+	7+	7+	8+
	BR-3	7+	7+	8+	7+	7+	8+	7+	7+	7+	7+
	Algeria	–	–	–	–	–	–	–	–	–	6+
	ASP-1	–	–	–	–	–	–	–	–	–	6+
	Israel	–	–	–	–	–	–	–	–	–	6+
	Iran	–	–	–	–	–	–	–	–	–	6+
I-2	AS-576	–	–	–	–	–	–	–	–	–	3+
I-3	I-747	–	–	–	–	–	–	–	–	–	2+
	Miss E	–	–	–	–	–	–	–	–	–	2+
I-4	277F	–	–	–	–	–	–	–	–	–	–
I-5	LB 12	–	–	–	–	–	–	–	–	–	–
I-6	M55	–	–	–	–	–	–	–	–	–	–
I-7	N525	–	–	–	–	–	–	–	–	–	–/1+
I-8	P40	–	–	–	–	–	–	–	–	–	3+
III	23-6	–	–	–	–	–	–	–	–	–	–
IV	SR-3	–	–	–	–	–	–	–	–	–	–
	B31	–	–	–	–	–	–	–	–	–	–
V	SMCA	–	–	–	–	–	–	–	–	–	–
VI	Y32	–	–	–	–	–	–	–	–	–	–
VII	MQ-1	–	–	–	–	–	–	–	–	–	–
VIII	EA-1	–	–	–	–	–	–	–	–	–	–
IX	CN-5	–	–	–	–	–	–	–	–	–	–
X	AES-1	–	–	–	–	–	–	–	–	–	–
XI	MQ-4	–	–	–	–	–	–	–	–	–	–
XII	DU-1	–	–	–	–	–	–	–	–	–	–/1+
XIII	CC-1	–	–	–	–	–	–	–	–	–	–
XIV	EC-1	–	–	–	–	–	–	–	–	–	–
XV	I-92	–	–	–	–	–	–	–	–	–	–/1+
XVI	CB-1	–	–	–	–	–	–	–	–	–	–
XVII	DF-1	–	–	–	–	–	–	–	–	–	–
XVIII	TN-1	–	–	–	–	–	–	–	–	–	–
XIX	PUP-1	–	–	–	–	–	–	–	–	–	–
XXI	I-25	–	–	–	–	–	–	–	–	–	1+

[a] Protein content of antigen was 0.5 μg/well.
[b] Protein content of antibody was 5 μg/ml.
[c] Conventional polyclonal antibody purified from mouse antiascites.
[d] Absorbance at 490 nm: 0.0–0.1 (–); 0.1–0.2 (1+); 0.2–0.4 (2+); 0.4–0.6 (3+); 0.6–0.8 (4+); 0.8–1.0 (5+); 1.0–1.2 (6+); 1.2–1.4 (7+); 1.4–1.6 (8+).

nonspecifically reacted with honeybee spiroplasma (AS-576), corn stunt spiroplasma (strains I-747 and Miss E), P40 spiroplasma (P40) of the group I, and I-25 spiroplasma (I-25) of the group XXI (Table 3).

DISCUSSION

Monoclonal antibodies with high specificity against *S. citri* (Maroc) have been produced by the hybridoma technique. To our knowledge, this is the first report on monoclonal antibodies produced against *S. citri* (Maroc). The methods used to produce monoclonal antibodies in this study ensure molecular homogenity. Thus, unlimited quantities of uniform immunoglobulin to a specific antigenic determinant of *S. citri* (Maroc) could be available for future research.

Conventional polyclonal antisera against spiroplasmas have been used for serotyping different strains. However, variability in results were encountered when different serological methods were employed or different animals were immunized (5). Therefore, when using polyclonal antisera, there is no accurate way to measure the differences in the specificity of antibody population of each antiserum collected. Our data clearly demonstrate that monoclonal antibodies can be used to overcome these problems.

The ELISA technique, using conventional heterogeneous antisera (polyclonal antisera), has been applied to detect *S. citri* in crude extracts of infected plants and insects (2,7) and to reveal the serological relatedness of spiroplasmas (1,18). Because of the existance of common antigenic determinants among spiroplasmas and the high sensitivity of ELISA, high ELISA readings (0.1–0.5) are frequently obtained between heterologous antiserum-spiroplasma combinations (18). If a plant was infected with more than one spiroplasma, unnecessary confusion in diagnosis or detection of a spiroplasma disease would certainly be encountered. Combining the sensitivity of ELISA with the specificity of the monoclonal antibody would provide a powerful diagnostic method. Monoclonal antibody that will not cross react with related spiroplasmas can be useful for disease diagnosis and may also be used for quick taxonomic differentiation.

The hybridoma clones that we selected possess a great discriminatory capacity for differentiating spiroplasma strains even within the same species or serogroup. In the antibody specificity study, the existance of serological differences among the strains of *S. citri* from different geographic areas was demonstrated for the first time. All the monoclonal antibodies tested reacted specifically with all of the nine native strains (C189, CB-1, Arizona, Aceratagallia, SCC, Scaph, L163, B106, and BR-3) and one North African strain (Maroc), but did not react with the four foreign strains: one from Iran (Iran), one from Algeria (Algeria), and two from Israel (ASP-1 and Israel).

Spiroplasmas have recently been isolated with increased frequency from various sources, paricularly from the arthropods (4,8,9). Research workers (9,20) have speculated that spiroplasmas may represent a great number of species among the prokaryotic organisms. Therefore, it might become increasingly difficult to differentiate spiroplasma isolates or strains by serotyping without highly specific techniques. The highly specific monoclonal antibodies should be able to reveal the phylogenetic relationships among these isolates, and their unique serological characters might provide a basis for differentiating them.

The monoclonal antibody technique can also be applied to the production of antibodies against the uncultivable MLOs that are responsible for more than 200 yellows diseases of plants. This technique allows the removal of inevitable contaminants of plant antigens in the antibody preparation. Monoclonal antibodies against aster yellows MLO have recently been produced in our laboratory (16). Monoclonal antibodies to MLOs or spiroplasmas should play a key role in diagnostic immunology of these organisms.

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