Comparison of Immunized Mouse Ascites Fluids and Rabbit Sera in Serological Tests of Two Spiroplasmas

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ABSTRACT

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Mouse ascites fluid prepared against two plant pathogenic spiroplasmas distinguished two spiroplasmas more clearly than did rabbit sera. Ascites fluid was induced with Freund's complete adjuvant in immunized adult female $C_{57}BL/6$ mice. *Spiroplasma citri* (*S. citri*) and the corn stunt spiroplasma (CS-747, New Jersey isolate) were used as antigens. Deformation, metabolic inhibition and growth inhibition tests were used to assess the relationship between *S. citri* and CS-747 and the immunospecificity and sensitivity of ascites fluid and antiserum. In the growth inhibition test, ascites fluid distinguished *S. citri* and CS-747 completely, reflecting a high degree of specificity. Both spiroplasma deformation and metabolic inhibition tests showed similar results although a small degree of cross reaction between the two spiroplasmas was detected. When rabbit sera were used for the same tests the titers for homologous and heterologous reactions were less distinct. The method of producing antibodies from mouse ascites fluid also offers several advantages: it requires less antigen to produce high titer of antibodies in the ascites fluid; the immunization time is relatively short; the animals can be held for continual antibody production; and only a few animals are required so that a large holding space is not needed.

Spiroplasmas were first recognized as unusual microorganisms in plants in 1972 (6,7,14). The helical morphology and the motility of spiroplasmas distinguish them from other mycoplasmas. In 1976, a new family, Spiroplasmataceae, included in the order Mycoplasmatales and the class Mollicutes was recommended by Skripal and accepted by the International Committee on Mycoplasma Taxonomy (7). At present, *Spiroplasma citri* and the corn stunt spiroplasma are the only two isolates proved to be plant pathogens (1,12,14) and *S. citri* is the only species that has been named.

Recent rapid advances in the isolation of spiroplasmas and the discovery of new strains (2,3,5) have resulted in confusion in identifying different isolates. It has been suggested that comparative studies of spiroplasmas should be carried out by restriction-enzyme patterns, SDS gel patterns, deoxyribonucleic acid hybridization, and various serological tests (8). Some of these methods require special facilities and are time-consuming. Serology could be a relatively easy technique for quick identification. However, in our previous serological tests, rabbit antisera prepared against different isolates of spiroplasmas showed various degrees of cross immunological reactions (1,18,20). Individual rabbit serum also exhibitis different specificity which makes the interpretation of tests quite difficult. Therefore, an attempt was made to find an antibody source more specific and more sensitive than rabbits. Previous reports have shown that large amounts of antibodies are found in ascites fluid of mice and guinea pigs (10,15,19). Mouse ascites fluid containing antibodies could be induced by several intraperitoneal injections of antigens and Freund's complete adjuvant (11) or by tumor inducing viruses (16). This study compares the sensitivity and specificity of immune mouse ascites fluid with that of rabbit antisera in the several serological tests to distinguish *S. citri* and corn stunt spiroplasma.

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MATERIALS AND METHODS

Experimental animals. Eight-week-old female $C_{57}BL/6$ mice (purchased from Microbiological Associates, Bethesda, MD) were housed in groups of five per cage. Each group of animals was immunized with one spiroplasma antigen.

Preparation of antigens (AG)—spiroplasmas. *S. citri* (Morocco Strain, ATCC 27556) (14) and corn stunt spiroplasma (New Jersey isolate CS-747, ATCC 29051), (1) were used as antigens in the experiment. Spiroplasmas were cultured in C-3G medium (9). In order to obtain spiroplasma cells, 100 ml of spiroplasma culture (3×10^8 cells/ml) was inoculated into 400 ml of sterile C-3G medium. After 3–5 days, when the growth of spiroplasma had reached its log-phase stage, the cells were harvested by centrifugation (27,000 *g* for 20 min) and washed three times with phosphate buffered saline (PBS, pH 7.0). The pellet was then suspended in 10 ml of PBS solution. Spiroplasma cells were subjected to freezing and thawing three times, homogenized with a teflon homogenizer (Sorvall Co.) at 1,500 rpm. The homogenate was stored at −20 C for use as antigen.

Prior to immunization of the animals, antigen was emulsified with Freund's complete adjuvant (AG-Adj) at 1:1 (v/v) ratio in a 50-ml centrifuge tube with a vortex mixer. PBS solution without spiroplasma antigen was used for injection of control animals.

Induction and collection of immune mouse ascites fluid (AF). All inoculations were given intraperitoneally according to the schedule shown in Table 1. Mice were examined every 2–3 days for the appearance of a swollen abdomen (Fig. 1). Three days after the last injection, the AF was withdrawn from the peritoneal cavity with a 0.723-mm-diameter (21-gauge) needle. The needle was inserted a minimal distance into the peritoneal cavity at a position where fluid had accumulated. The collected fluid from each group of five mice was pooled and was centrifuged at 1,000 *g* to remove the fibrin clot and peritoneal cells. The clear supernatant was sterilized by filtering successively through 0.8- and 0.45-μm Millipore filters and then stored at −20 C.

Production of antisera (AS) from rabbit. Rabbits were injected intramuscularly at weekly intervals as shown in Table 1. The blood was collected from the ear veins and incubated in a 37 C water bath for 1 hr. It was then centrifuged at 1,000 *g* for 10–15 min to sediment the red blood cells. The supernatant sera were pooled and stored at −20 C.

Titration of antisera and immune mouse ascites fluid. Modified spiroplasma organism deformation (13,21), modified metabolic inhibition (17), and growth inhibition (4) tests were employed to study the serological relationship of *S. citri* and CS-747 spiroplasmas. The cell concentration used for different tests varied and was predetermined before each test. Cell titer was measured by the method described by Liao and Chen (9).

Deformation test (DF). The test was carried out in accordance with the following protocol. Two-tenths milliliter of AF (or AS) was added to the first tube which contained 0.8 ml of C-3G medium. Five-tenths milliliter of the mixture was transferred from the first tube to the next tube which contained 0.5 ml of C-3G medium. Serial transfers were made so that each tube contained 0.5 ml of mixture and the sets resulted in a serial dilution of AF (or AS) at 1:10, 1:20, 1:40, ...1:10,240. The initial cell concentration used as antigen was predetermined at 6×10^6 helices per milliliter. Five-tenths milliliter of this antigen was added to each of the tubes containing a mixture of serial diluted AF (or AS). After 1 hr of incubation at 30 C, samples (5 μl each) were taken from all the tubes. The cell number and morphology were observed under a dark-field microscope. The titer of AF (or AS) was determined as the highest dilution tube in which the concentration of undeformed cells was less than or equal to one-half that of the controls. The control tube contained 0.5 ml of cell culture (3×10^6 cells per milliliter) without AF (or AS) added. In addition, a set of control tubes were made with serially diluted AF or AS from the control animals (anti-PBS).

Metabolic inhibition test (MI). Serial twofold dilutions of AF (or AS) in cell culture were the same as in the DF test except the total final volumes were 2 ml in each tube and the cell concentration was much lower (2×10^4 cells per milliliter). The titer was determined to be the reciprocal of the highest dilution of AF (or AS) that would not permit multiplication of spiroplasmas as shown by acid production and which changed the red color of the phenol-red indicator in the medium to yellow. The control tube contained only 2 ml of cell culture (2×10^4 cells per milliliter). An additional control set was made with serially diluted AF (or AS) from control animals (anti-PBS).

Growth inhibition test (GI). Two and five-tenths milliliters of C-3G medium containing 0.8% Ionagar, No. 2 (Oxoid, Chicago, IL) was poured into each 50 × 9-mm petri plate (Falcon Plastics, Oxnard, CA 92030). After the culture media solidified, 0.2 ml of spiroplasma suspension (10^5 cells per milliliter) was transferred into each plate. Care was taken to insure that the inoculum covered the entire agar surface and time was allowed for the medium in each plate to soak up the inoculum. A sterile filter paper disk (0.65 cm in diameter) saturated with 5 μl of AF (or AS) was air-dried and carefully laid onto the agar surface at the center of each plate. Three duplicates of each AS-AG pair were prepared. The plates were incubated at 30 C for 10–14 days and were observed for spiroplasma growth. The inhibition zone with no spiroplasma growth, if present, was measured with the aid of a dissecting microscope.

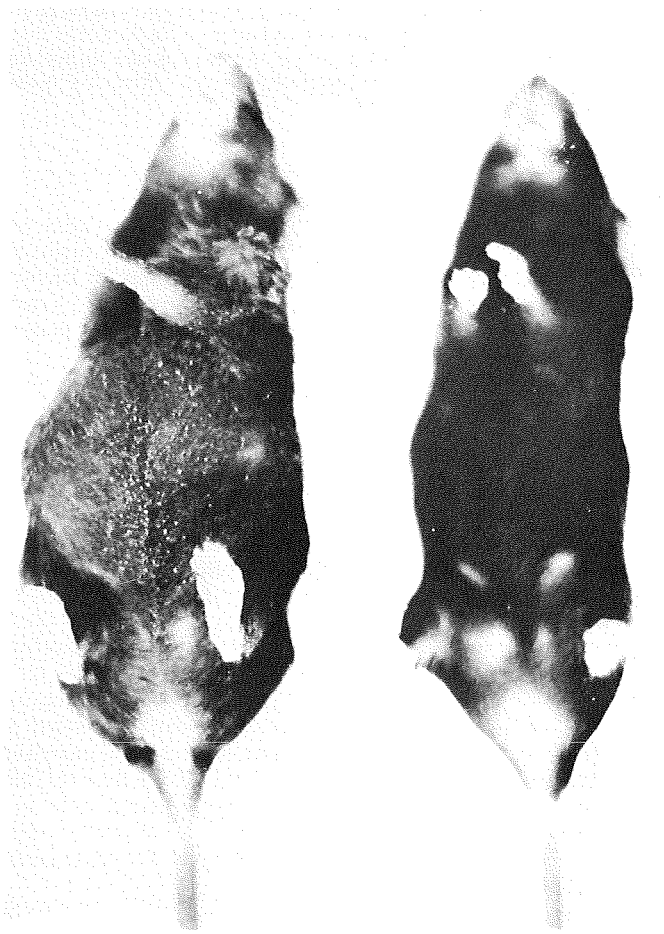

Fig. 1. Symptoms of ascites induced in $C_{57}BL/6$ mice by injecting Freund's complete adjuvant. Treated mouse on left shows a distended abdomen.

RESULTS

Approximately 2–8 ml of AF could be withdrawn from each inoculated mouse at each harvest. Individual mice varied markedly in the time (3–6 wk) required to produce appreciable amounts of AF. In order to protect AF from bacterial contamination, the harvested AF was filtered through 0.8- and 0.45-μm Millipore filters. This resulted in a loss of some fluid. It was advantageous to pool the AF from the same group of mice because it reduced the deviation of immuno-responses among the individual animals. For each group of mice, 10–25 ml of filtered AF were obtained.

A comparison of the degree of specificity and sensitivity of the two antibody sources as determined by DF and MI tests can be seen

TABLE 1. Immunizing schedules for producing spiroplasma immune mouse ascites fluid and antiserum of rabbit

	Dosage per application			
	Rabbit (ml)		Mouse (ml)	
Day	Adjuvant	Antigen	Adjuvant	Antigen
0	2.5	2.5	0.25	0.25
7	...	5.0	...	0.5
14	...	5.0	0.25	0.5
17	...	5.0	0.25	...
24	...	...	0.25	0.5
30	...	5.0	harvest	0.1[a]
35	...	...	harvest	0.1[a]
45	harvest	...	harvest	0.1[a]

[a] Postharvest subcutaneous injections of antigen were made to maintain the antibody titer.

in Tables 2 and 3. Mouse AF showed great differences between the homologous and heterologous titers, whereas with rabbit AS the differences between homologous and heterologous titers were less dramatic.

The differences in specificities between mouse AF and rabbit AS in GI tests are shown in Table 4. In GI tests, the AF against *S. citri* inhibited its homologous antigen in a 6.75 mm-wide zone. The anti-CS-747 AF inhibited CS-747 spiroplasma from forming colonies in a 9.35 mm-wide zone. In both heterologous AF-AF reactions, growth inhibition zones were not detected. The colonies formed by the spiroplasmas in heterologous tests with AF reached the edge of the filter paper disks, and the number of the colonies formed equaled that in the control plates.

In DF tests, the titer of both anti-CS-747 AF and anti-*S. citri* AF against their homologous spiroplasma antigens were 20,480. When anti-*S. citri* AF as used against CS, the titer was 40. The heterologous titer of anti-CS-747 AF against *S. citri* was 80. On the other hand, when rabbit antisera were used, the homologous AS titer against both organisms was 10,240. The heterologous titers of AS against *S. citri* and CS-747 were 640 and 1,280, respectively.

The results of MI tests showed the same trend as those in the spiroplasma deformation tests. In MI tests, homologous titers of AF against *S. citri* and CS-747 were 10,240 and 20,480; the heterologous titers were 80 and 160. The homologous titers of AS against *S.* citri and CS-747 were 10,240 and 5,120 and the heterologous were 640 and 2,560, respectively. The titers of control AF in both DF and MI tests were less than 10 which was the lowest dilution tube in the series, and thus showed no inhibition.

DISCUSSION AND CONCLUSIONS

Previous reports (1,20) on the serological relationship between *S. citri* and CS-747 showed that cross-reactions occurred in the heterologous reactions at only a fourfold difference in titer from homologous, not a sufficient difference for reliable separation of the two spiroplasmas. In this study, AF proved clear evidence of differences as demonstrated in the results of the various serological tests, especially in the GI test.

Two factors affect the results of growth inhibition (GI) test of spiroplasmas. First, the growth rates of different spiroplasmas may vary greatly. As shown in Table 2, the homologous inhibition zone of CS-747 is greater than that of *S. citri*. This is because for a serum of given antibody titer, the longer the time it takes for a spiroplasma cell to form a visible colony, the wider the gradient of growth-inhibiting antibody concentration from the center disk will be. The second factor is the concentration of spiroplasma cells used to inoculate the plates. As expected in antibody-antigen reactions, an excess of antigen could reduce the titer of the antibody to zero. Thus, if an excess of spiroplasma cells was added to the plate, either a very small or no inhibition zone would be observed around the disk. This means the GI test requires a preliminary determination of antigen concentration. Even though the determination of the right cell concentration and the preparation of agar plates make the GI test more laborious, this technique shows more specificity than DF and MI tests, especially when AF is used.

The DF test offers quick results, but the procedure can be very time consuming when several parallel and reciprocal tests are to be read. It is important that the spiroplasma culture used as the antigen in the deformation test (DF) be in its log-phase growth. The spiroplasma should have good helicity for the accurate observation and determination of the "deformed cells." Test results read by different individuals may deviate due to biased interpretation.

In the metabolic inhibition (MI) test better results were obtained by carrying out the serological procedures in small test tubes. When clumping of spiroplasma cells occurs it is difficult to make an accurate dilution series with very small quantities of medium. Although the use of test tubes requires more culture medium and AF (or AS) dilution of antigen is more accurate giving more satisfactory results.

The method of producing antibodies from mouse ascites fluid offers several advantages. Large holding space is not needed for rearing mice. Mice require less antigen to produce high titers of antibodies in the ascites fluid. The immunizing time for mice is relatively short compared to that for rabbits. Individual mice, although small, can yield as much as 8–10 ml of AF weekly. In addition, the titer of rabbit AS was found to vary considerably depending upon the individual rabbit. There was very little variability in the AF taken from the mice, presumably because individuals of the inbred $C_{57}BL/6$ strain have an identical genetic background. Titer variability that was found was eliminated by combining the AF from all mice immunized with the same antigen. This makes tests done at different times more consistent.

The immune system in various kinds of animals is similar. Immunoglobulin G is dominant in both AF of mouse or AS of rabbit. This study indicates rabbits produce antibodies against relatively wider range of antigenic determinants of spiroplasma than does the immune system of mouse. Mouse antibodies in AF appear to detect specific antigenic determinants which permit one to differentiate the two plant pathogenic spiroplasmas clearly by the simple GI test. It is our hope that mouse AF will prove more useful than rabbit antisera for differentiating other spiroplasma strains, particularly when the GI test is used.

TABLE 2. A serological comparison between *Spiroplasma citri* and corn stunt spiroplasma (CS-747) using immune mouse ascites fluids and rabbit antisera in modified spiroplasma deformation tests.

Antigen/ antibody	Mouse ascites fluid			Rabbit antiserum		
	S. citri	CS-747	Control (PBS)	*S. citri*	CS-747	Control (PBS)
S. citri	20,480	80	<10	10,240	640	10
CS-747	40	20,480	<10	1,280	10,240	10

TABLE 3. A serological comparison between *Spiroplasma citri* and corn stunt spiroplasma (CS-747) using immune mouse ascites fluids and rabbit antisera in modified metabolic inhibition test

Antigen/ antibody	Mouse ascites fluid			Rabbit antisera		
	S. citri	CS-747	Control (PBS)	*S. citri*	CS-747	Control (PBS)
S. citri	10,240	80	<10	10,240	640	10
CS-747	160	20,480	<10	2,560	5,120	10

TABLE 4. A serological comparison of the inhibition zone (mm)[a] between *Spiroplasma citri* and corn stunt spiroplasma (CS-747) using immune mouse ascites fluids and rabbit antisera in growth inhibition test

Antigen/ antibody	Mouse ascites fluid			Rabbit antiserum		
	S. citri	CS-747	Control (PBS)	*S. citri*	CS-747	Control (PBS)
S. citri	6.8	0	0	7.3	2.7	0
CS-747	0	9.4	0	4.3	12.3	0

[a]The inhibition zone, an average of five measurements per plate, was measured from the margin of the disk to the edge of the zone.

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