Partial Characterization of the Principal Soluble Antigens Associated with the Coronavirus of Transmissible Gastroenteritis by Complement Fixation and Immunodiffusion

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A microtiter complement fixation (CF) test to detect transmissible gastroenteritis (TGE) viral antigen was developed, using TGE hyperimmune pig serum as an antibody source. Sera from TGE convalescent pigs did not fix complement by this test. Maximal virus and soluble antigen (SA) titers were obtained 36 to 48 h after inoculation of swine testes cells. Cell-associated virus and SA titers were higher than those in the culture fluid, which had to be concentrated 20× before use as antigen in agar immunodiffusion tests (ID). By sucrose density-gradient centrifugation, the SA had a buoyant density of 1.10 g/ml and could be separated from the virus that banded in the 1.19-g/ml region. Virus and SA from three different isolates of TGE had the same buoyant densities. Heating and proteolytic enzyme digestion established the protein nature of the SA. As assayed by CF and ID, there were stability differences between crude and purified preparations of SA. Antibody prepared in rabbits against the SA neutralized the TGE virus.

One porcine disease that is of increasing economic importance is transmissible gastroenteritis (TGE) (2, 6). This is a very contagious disease which has a high mortality in newborn pigs. The causative virus has been identified as a single agent (3, 9, 11, 16, 17) with the characteristics of a coronavirus (9). Viruses of this group also infect humans, chickens, and rodents (9, 11). Immunological studies have been described for precipitating antigens, and a complement fixation test (CF) for avian and human coronavirus-associated antigens has been described (4, 7, 8, 15, 18). There is one report on the use of the agar gel immunodiffusion (ID)-precipitating test, (1) but none on the use of a CF to aid in the antigenic characterization of TGE coronavirus.

These experiments were designed: (i) to determine the feasibility of adapting the ID and CF tests to study TGE virus by using immune swine serum; (ii) to separate soluble antigens (SA) from the virion; (iii) to establish enzyme and heat stability of the SA; and (iv) to determine whether an immunological relationship exists between virus neutralization and anti-SA antibody.

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MATERIALS AND METHODS

Viruses. Three isolates of TGE coronavirus were used. The first was the Miller no. 3 as described by Tamoglia (14), and the other two were isolated from pigs sick with TGE in Minnesota (19) and Illinois (12). The Miller isolate had been passaged six times in a continuous line of swine testes (ST) cells according to the procedure of McClurkin and Norman (10), and the other isolates had been passaged 28 and two times, respectively. All viruses were grown in ST cells in 250-ml plastic bottles containing 25 ml of Earle basal media with Eagle minimum essential medium, prepared in Earle balanced salt solution, supplemented with 10 ml of a 100× vitamin solution, 20 ml of a 50× amino acid solution, 6 ml of a 200 mM L-glutamine solution (Grand Island Biological Co., Grand Island, N.Y.), and 0.5% lactalbumin hydrolysate media, and were seeded with 3 ml of 10⁵ plaque-forming units of virus (10).

For the growth curve study, the Miller isolate was used. Because of the 4- to 6-h eclipse phase associated with the growth of TGE virus in cell culture (20), the first assay for virus was made after this time interval. At 8 h after seeding and afterwards at 12- or 24-h intervals, two bottles were removed, frozen, and thawed to dislodge the cell sheet. Cells were separated from the media by centrifugation, washed with 10 volumes of phosphate-buffered saline (pH 7.2), resuspended in 5 volumes of phosphate-buffered saline, and ultrasonically disrupted at 0 to 5 C. This suspension was clarified by

centrifugation at approximately $12,000 \times g$ for 20 min, and the supernatant fraction was removed. The disrupted cells were resuspended in 5 volumes of phosphate-buffered saline and subjected to the same process. Both supernatant fractions were combined and assayed for virus by the plaque test in ST cells (10). This fraction was considered to be the cell-associated virus and antigen. The supernatant fluid was concentrated $20\times$ by membrane filtration by using XM-50 membranes (Amicon Corp., Lexington, Mass.), which have a cutoff of approximately 50,000 daltons.

To prepare virus for density-gradient centrifugation, it was necessary to concentrate TGE virus-infected cell culture fluids 50 to $100\times$, and this was accomplished as above by membrane filtration. The sonically disrupted cell supernatant fraction was usually included in the filtration.

Antiserum. For the preparation of hyperimmune serum, a 10% homogenate of intestinal tissue from a pig infected 48 h previously with TGE virus was concentrated by layering 10 ml of the homogenate on 20 ml of a 30% sucrose solution layer and centrifuging it at 25,000 rpm in an SW25.1 rotor (Beckman Instruments, Inc., Fullerton, Calif.) for 4 h. The virus-containing band was separated, passed through a 220-nm filter for sterilization, and used as antigen. Pigs were inoculated intravenously with 25-ml aliquots three times at 3-week intervals. The pigs were bled for serum 2 weeks after the last injection. Sera were collected from pigs at 55 to 110 days after infection with TGE.

ID precipitin test. Agar plates were prepared by using 1% agarose in 0.15 M borate buffer (pH 8.6) and 4% polyethylene glycol (molecular weight 6,000). Wells 2 mm in diameter and 2 mm apart were cut, and the test was controlled by using an extract of uninfected ST cells and normal pig serum.

It was necessary to concentrate the TGE virus-infected cell culture fluids 10 to $20\times$ by membrane filtration to produce a visible precipitin line, whereas the cell-associated antigen could be used without concentration.

CF test. The test used "microtiter" volumes of 0.025 ml for antigen, hyperimmune pig serum previously heated to 56°C for 30 min, and guinea pig complement diluted to contain five 50% hemolytic units of complement in the presence of antiserum or antigen. Unheated 1% bovine serum was used to enhance CF and reduce pig serum procomplementary activity. After overnight incubation at 5°C , 0.025 ml of optimally sensitized 0.6% sheep erythrocytes was added, followed by incubation at 37°C for 30 min. The unlysed cells were allowed to settle, and the end point titers were determined by comparison to wells containing 25, 50, and 75% of the cell concentration used in the test. The well with the highest dilution of antigen or serum having 50% or less hemolysis was considered to be the end point. A chessboard titration was used to establish end points for serum and antigen, and four units of antiserum and two units of antigens were used in subsequent tests.

Virus neutralization test. The procedure employed was the plaque reduction procedure, as de-

scribed by McClurkin and Norman (10).

Density-gradient centrifugation. Sucrose solutions were prepared in phosphate-buffered saline with the following densities: 1.05, 1.10, 1.15, 1.20, and 1.25 g/ml. Three milliliters of the 1.25-g/ml sucrose solution was placed in the bottom of the tube, and 2 ml of descending lighter sucrose solutions was placed on top. After 4 h of equilibration, 1 ml of concentrated virus preparation was placed on top, and the tubes were centrifuged in an SW41 rotor at 40,000 rpm for 18 h at 5°C . One tube in each run contained 1.10- and 1.20-g/ml buoyant density marker beads. After completion of the run, 12 0.5-ml samples were removed from the top of the tube by using an automatic pipette, and the remainder of the gradient was removed by puncturing the bottom and collecting 0.5-ml fractions. The density of the various sucrose fractions was determined refractometrically. All fractions were tested for the presence of virus by titration in ST cells. SA was assayed by ID and CF tests. Serologically active fractions were concentrated by vacuum dialysis against 0.85% saline and phosphate-buffered saline to the original volume of sample used in the centrifugation. The SA and the virus fraction were centrifuged separately by the same method.

For subsequent CF and ID tests and enzyme experiments, SA that had been recentrifuged twice on the gradient was considered to be purified, and the original concentrate of infected cell culture fluids and disrupted ST cells was considered to be crude antigen.

SA. For rabbit inoculation, SA was prepared by recycling the pooled sucrose density gradient fractions three through eight times. This fraction had an ID titer of 1:4 to 1:8 and was mixed with an equal volume of Freund complete adjuvant. Toe pads of adult New Zealand white rabbits were injected twice at weekly intervals with 0.1 and 1 ml intramuscularly, followed by 0.2 ml intravenously without adjuvant at bi-weekly intervals for three additional injections. Rabbits were bled for serum 7 to 10 days after the last injection. All sera were inactivated at 56°C for 30 min prior to the virus neutralization tests.

Heat sensitivity. Aliquots of SA were placed in thin-walled glass tubes, capped, immersed in a water bath at the appropriate temperature for 15 min, and immediately cooled in an ice-water bath.

Enzymes. The following enzymes were used to test the stability of the SA: pepsin, $2\times$ crystalline, lot 86B-3010 (Sigma Chemical Co., St. Louis, Mo.); lipase (Sigma Chemical Co.); lyophilized hog pancreas, lot L-2253; papain, lot PAP 7KB (Worthington Biochemicals Corp., Freehold, N.J.); trypsin, lot 0461211 (Boehringer, Mannheim, Germany); deoxyribonuclease, lot 7413305 (Boehringer); ribonuclease, lot 7333126/1 (Boehringer). Enzymes were reactivated as required, and reactions were performed at a pH appropriate for each. An enzyme weight-to-substrate nitrogen ratio of 1:25 was used with an incubation of 2 h at 37°C . Pepsin activity was terminated by adjusting the pH to 7.2, and soybean trypsin inhibitor was used to stop tryptic activity.

FIG. 1. Growth curve of the Miller isolate of TGE virus in ST cells, as assayed by virus infectivity, CF, and ID.

RESULTS

The data in Fig. 1 show that virus growth was detected at 12 h after seeding and reached maximal growth between 36 and 48 h. This was followed by a decline at 120 h, the last time interval tested. Most of the virus appeared to be associated with the cellular fraction of the culture, and the virus titers were 1 to 2.5 log₁₀ higher than the cell-free medium.

Complement-fixing and precipitating antigens were first detected in the 24-h growth sample. Higher concentrations of antigen were associated with the cellular fraction by both CF and ID tests from 24 to 72 h of growth, and the CF titer was generally higher than the ID titers. In the 96- and 120-h samples CF and ID titers of the cell-associated antigen were equal, but CF antigen could not be detected in the media.

The ID tests of a typical growth curve are shown in Fig. 2. The intensity of the precipitin bands is generally more distinct with the cell-associated antigen, and, as seen in the 48 h sample, a double line may be visible.

After sucrose density-gradient centrifugation (Fig. 3), the SA, as assayed by ID, was usually found in fractions 3 to 6, corresponding to a density of 1.10 g/ml. In contrast, CF activity was found in fractions 7 to 8. Recycling the positive fractions 3 to 8 or virus fractions 15 to 17 separately did not alter the location of the density zones for SA or virion. The location of the SA and virus in the sucrose gradient was the same for the Minnesota and Illinois isolates.

Nucleases had no discernible effect upon either the crude or purified SA, as assayed by ID or CF tests (Table 1). Porcine lipase reduced

FIG. 2. Agar ID precipitin patterns of TGE virus grown in ST cells. Hyperimmune serum is in the central well and in the 10-o'clock well in the 12-, 24-, 36-, 48-, and 72-h samples. A known positive TGE antigen is in the 10-o'clock well in the 8-h sample. Undiluted antigen is in the 12-o'clock position in the outer well and two-fold dilutions in the outer well. Abbreviations: S, cell-free supernatant fraction; C, cell-associated antigen.

FIG. 3. Sucrose density-gradient centrifugation of the Miller isolate of TGE virus, as assayed by virus infectivity, CF, and ID. Symbol: +, positive agar ID precipitin test.

TABLE 1. Effect of enzymes on the SA of TGE virus

Enzyme	Crude antigen		Purified antigen	
	ID	CF	ID	CF
Deoxyribonuclease	Resistant ^a	Resistant	Resistant	Resistant
Ribonuclease	Resistant	Resistant	Resistant	Resistant
Lipase (porcine)	Reduced	Resistant	Sensitive ^b	Sensitive
Papain	Sensitive	Reduced ^c	Sensitive	Reduced
Pepsin	Sensitive	Reduced	Sensitive	Sensitive
Trypsin	Sensitive	Sensitive	Sensitive	Sensitive

^a Resistant, No change in dilution titer.

^b Sensitive, No detectible titer.

^c Reduced, Decrease in dilution titer of 1:2 or greater.

the ID titer of the SA and eliminated the ID titer of the purified antigen. In contrast, lipase did not alter the CF titer of the crude antigen, and only in the purified antigen was the CF titer eliminated. The three proteolytic enzymes (pepsin, papain, and trypsin) eliminated the ID titer of both crude and purified antigens, whereas with trypsin CF titers were eliminated with both preparations of antigen.

As assayed by the CF test, the crude antigen was only reduced in titer by pepsin, whereas the purified antigen CF titer was eliminated (Table 1), and the CF antigen was sensitive to the action of trypsin.

Table 2 shows the effect of temperature upon the antigen. Heating to 56 C was sufficient to eliminate the ID titer of the crude antigen. The ID titer of the purified antigen was resistant at 65 C, but reduced at 80 C and eliminated at 100 C. As assayed by CF test, both the crude

TABLE 2. Effect of temperature on the SA of TGE virus

Temp (C)	Crude antigen		Purified antigen	
	ID	CF	ID	CF
37	Resistant ^a	Resistant	Resistant	Resistant
56	Sensitive ^b	Resistant	Resistant	Resistant
65	Sensitive	Reduced ^c	Resistant	Reduced
80	Sensitive	Sensitive	Reduced	Reduced
100	Sensitive	Sensitive	Sensitive	Sensitive

^a Resistant, No change in dilution titer.

^b Sensitive, No detectible titer.

^c Reduced, Decrease in dilution titer of 1:2 or greater.

and purified antigens were resistant at 56 C. Heating the antigen to 65 only reduced the CF titer of the crude antigen preparation, but at 80 and 100 C CF activity was eliminated. The CF titer of the purified antigen was reduced at 65 and 80 C and eliminated at 100 C.

Attempts to detect CF antibody in convalescent sera obtained from pigs infected under experimental conditions with TGE were generally unsuccessful, in spite of the presence of high-titer, virus-neutralizing antibody (Table 3).

Rabbit antiserum prepared against SA had a 50% plaque reduction titer of 1:160, and virus-neutralizing antibody was not detectable with the pre-inoculation serum.

DISCUSSION

A CF test for TGE employing hyperimmune porcine serum has been developed. This adds porcine TGE coronavirus to the other coronaviruses, human (7), chicken (13), and mouse (4), to which a CF test has been successfully applied. It was not possible to use the CF test with swine convalescent from TGE (Table 3), and it may be necessary to use the indirect CF procedure (13), which generally is more sensitive, to detect CF antibody in these pigs. However, the application of this test has given us another parameter to study the antigens of TGE virus and, in conjunction with the ID procedures, has clearly demonstrated the separation of SA from the virion by using sucrose density-gradient centrifugation.

The density-gradient results (Fig. 2) indicate the presence of at least two SA associated with TGE, since the peak of CF activity does not coincide with that of the ID activity. Further evidence of two SA occurs in Fig. 2 in the 48-h sample, the growth curve of this virus that clearly shows two distinct precipitin bands in ID. The enzyme and heat stability data in Tables 1 and 2 also support the presence of two antigens, since differences in heat stability and sensitivity to enzymes are seen in SA assayed by ID and CF tests. The sensitivity of the SA to

lipase may suggest a lipoprotein-like structure. Unfortunately, a crystallized lipase free of proteolytic enzymes is not available, and the sensitivity may be due to contaminants of trypsin, since the SA is inactivated by this enzyme (Table 1). Kaye et al. (9) were unable to liberate CF antigen from human coronavirus by ether or ether-Tween treatment, suggesting that the antigen is closely associated with the virion. Our data (Fig. 1) also suggest that the SA is primarily cell associated, and the CF and ID titers of the cell-associated antigens parallel the virus. It is possible that the preparation and concentration of infected fluids and disruption of ST cells was sufficient to liberate SA. Differences between effects of enzymes and heat on crude and purified viral antigens are in partial agreement with those reported by Kaye et al. (9), who found that purified human coronavirus OC-43 was more sensitive to heat and trypsin action than were crude preparations. Our crude SA as assayed by CF or ID was more sensitive to heat than was purified SA; however, in agreement with Kaye et al. (9), our purified SA was more sensitive than crude SA to enzyme action.

Of importance is the finding that rabbit serum prepared against the SA had significant virus neutralization titers of 1:160. This is evidence that the SA is directly associated with the virion, and further studies applying immune-electron microscopy are required to establish the relationship of SA to the virion.

Our finding that three TGE virus isolates could be banded by sucrose-density centrifugation in the 1.19- to 1.20-g/ml region is in conformity with that of Witte et al. (17). That the SA of each isolate has the same isopycnic point is not surprising, since immunological distinctions among TGE viruses have not been reported.

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TABLE 3. TGE virus neutralization and CF titers of serum from pigs convalescent and hyperimmunized to TGE virus

Convalescent pig no.	Virus-neutralizing titer ^a	CF titer
46	1:300	Neg. ^b at 1:10
47	1:300	Neg. at 1:10
48	1:250	Neg. at 1:10
49	1:180	Neg. at 1:10
50	1:250	Neg. at 1:10
51	1:250	Neg. at 1:10
52	1:1,000	Neg. at 1:10
53	1:190	Neg. at 1:10
Hyperimmune	1:10,000	1:40
Normal	Neg. at 1:10	Neg. at 1:10

^a Dilution of serum for 50% plaque reduction.

^b Neg., Negative.

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