IMMUNOCHEMICAL STUDIES OF FOOT-AND-MOUTH DISEASE

V. Antigenic Variants of Virus Demonstrated by Immunodiffusion Analyses with 19S but not 7S Antibodies

By K. M. COWAN, Sc.D.

(From the Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, United States Department of Agriculture, Greenport, New York 11944)

(Received for publication 2 October 1968)

Immunodiffusion procedures provide extremely powerful tools for the resolution of complex antigen-antibody systems. The antisera utilized in such studies are almost always prepared by intensive immunization so that the antibody is primarily of the 7S class. A review of the literature has not revealed the use of 19S antibody for antigenic analysis in immunodiffusion procedures, or a comparison of 7S and 19S antibodies for this purpose.

Preliminary studies demonstrated that sera collected from guinea pigs 7 days postinfection (DPI) with foot-and-mouth disease virus (FMDV) gave two precipitin bands with a purified FMDV preparation in Ouchterlony tests while sera collected about 30 DPI gave a single band (1). The antibody demonstrable in the sera of FMDV-infected guinea pigs between the 4th and 8th DPI is almost solely of the 19S class, while that occurring after about 30 DPI is primarily 7S class[1] (2, 3). Similar observations on the nature of the antibodies produced were made with cattle (2, 4) and swine (5) infected with FMDV.

The present studies were undertaken to obtain additional information on the antigenic characteristics of FMDV and to compare 19S antibody-containing sera with 7S antibody-containing sera for these analyses. The results obtained demonstrated that 19S antibody could discriminate between three antigenic variants of FMDV and enzyme-treated virus preparations that could not be differentiated by 7S antibody in immunodiffusion analyses.

Materials and Methods

Virus.—Foot-and-mouth disease virus, type A, strain 119 (FMDV, A-119), was used in these studies and was grown in cultures of baby hamster kidney cells (BHK-21, clone 13 of MacPherson and Stoker) (7), or was obtained from the vesicular fluid from the footpads of guinea pigs infected with the virus. The various preparations of FMDV, A-119, studied were grown and purified as follows:

1. Tissue culture high passage virus: Virus grown and purified as described by Bachrach et al.

[1] Guinea pig antibodies of the 7S class are generally designated $7S\gamma_2$ and $7S\gamma_1$ (6). In the present study, no attempt was made to investigate these two antibodies individually and the activities studied will be attributed to antibodies of the 19S and 7S classes.

(8) and Polatnick and Bachrach (9) was provided by the Virus Production Unit of the Plum Island Animal Disease Laboratory. This virus had been passaged approximately 150 times in primary calf kidney cell cultures and finally grown in BHK-21 cultures for production purposes. These purified virus preparations contained from 1 to 6 mg of virus/ml and were shown to be homogenous by various physical and chemical procedures (8, 10, 11). These preparations are referred to as 140S-Hi-pass virus. The 140S designation refers to the sedimentation coefficient (*s* rate) of FMDV (12).

2. Tissue culture low passage virus: Virus from infectious bovine tongue epithelium was passaged seven times in calf kidney cell cultures and then grown in BHK-21 cells. The 140S virus particles were concentrated by $(NH_4)_2SO_4$ precipitation and purified by centrifugation into isodense CsCl (1.43 g/ml) as described previously (13, 14). This preparation is designated 140S-Lo-pass virus.

3. Large and small plaque-forming viruses: Stocks of large and small plaque-forming variants of FMDV, A-119, were isolated from high passage virus[2] and kindly supplied by Dr. J. Martinsen. These viruses were passaged two additional times in calf kidney cell cultures and grown in quantity in BHK-21 cultures. The virus in each preparation was purified in the same manner as the 140S-Lo-pass virus. These preparations of small and large plaque-forming virus are referred to as 140S-Sm.pl. and 140S-Lg.pl. viruses, respectively. Plaque assays of these purified viruses confirmed the plaque-producing characteristics reported by Martinsen.[2]

4. Guinea pig vesicular fluid virus:

(*a*) Virus from infectious bovine tongue epithelium was passaged 23–27 times in guinea pigs by intradermal inoculation of the footpads. Fluid collected from the vesicles that are produced within about 24 hr after inoculation provides an excellent source of virus. Virus was purified directly from the vesicular fluids by centrifugation into isodense CsCl. This virus preparation is designated as 140 S-GP virus.

(*b*) A guinea pig source of virus from tissue culture high passage virus was prepared by inoculating a group of guinea pigs with 140S-Hi-pass virus. Four passages were required for satisfactory adaptation and fluids obtained from the sixth passage were used for the preparation of purified virus as just described. This virus preparation is termed 140S-TC-GP.

The following is presented to summarize the various purified virus preparations used: *140S-Hi-pass virus* = FMDV, A-119, passaged approximately 150 times in calf kidney cell cultures and grown for purification purposes in BHK-21 cultures. *140S-Io-pass virus* = FMDV, A-119, from bovine tongue epithelium passaged seven times in calf kidney cell cultures and grown in BHK-21 cultures. *140S-Lg.pl. virus* = large plaque-forming FMDV, A-119, isolated from 140S-Hi-pass virus and grown in BHK-21 cultures. *140S-Sm.pl. virus* = small plaque-forming FMDV, A-119, isolated from 140S-Hi-pass virus and grown in BHK-21 cultures. *140S-GP virus* = FMDV, A-119, passaged 23–27 times by guinea pig footpad inoculation. *140S-TC-GP virus* = FMDV, A-119, of the 140S-Hi-pass type passaged six times in guinea pigs. *140S-b virus preparation* will be described under Results. It was isolated from 140S-Hi-pass virus, plaque purified in calf kidney cell cultures, and grown in quantity in BHK-21 cultures. It was purified in the same manner as 140S-Lo-pass virus.

Antisera.—Guinea pigs were infected by intradermal inoculation of the plantar pads with a guinea pig vesicular fluid source of FMDV, A-119. At various DPI, the guinea pigs were anesthetized with CO_2 and blood samples taken by cardiac puncture. Preliminary studies with individual sera indicated that blood collected on the same DPI could be pooled. The pooled serum was clarified by high speed centrifugation, heated for 30 min at 56°C and stored at −20°C. A relatively large pool of 7-DPI serum was prepared for use in the studies to be

[2] Martinsen, J. Biological characteristics of two plaque size variants of foot-and-mouth disease virus. Manuscript submitted for publication.

presented. The 7-DPI serum did not contain detectable antibody to the virus protein subunit (12S) antigen or to the virus infection-associated (VIA) antigen (14).

The term "hyperimmune" is applied to serum obtained from guinea pigs infected in the manner just described but after an additional inoculation of guinea pig-adapted virus given 10–12 wk after the initial infection. Blood samples were taken 10 days later and the serum collected and stored as just described. Hyperimmune sera contain antibody to the 140S, 12S, and VIA antigenic components (14).

Antiserum specific for the 140S particles has been described (15). In brief, this was prepared by immunization of guinea pigs with inactivated, purified 140S-Hi-pass virus emulsified in incomplete Freund's adjuvant. Blood samples were taken 35 days after a single inoculation of the vaccine. The pooled serum was absorbed with virus protein-subunit antigen (12S) to make the serum specific for 140S particles. This serum is termed anti-140S serum.

The ultracentrifugation procedure using a KBr-$NaNO_3$ gradient for separating 19S and 7S classes of antibodies was performed as described previously (3).

Serological Methods.—The Ouchterlony-type agar gel diffusion precipitin technique and the acridine orange-staining procedure, as applied to the study of FMDV, have been described (13, 14, 16). Immunoelectrophoretic analyses were performed by the general method of Scheidegger (17). In the present investigations, the various virus preparations were adjusted to a concentration of approximately 0.5 mg/ml for immunodiffusion assays. The assays used to determine the 140S content of the virus preparations were a quantitative complement-fixation procedure (18) and spectrophotometric examination at a wavelength of 258 mμ (8). It was necessary to dilute the anti-140S and hyperimmune sera because of their relatively high 7S antibody content and its rapid diffusion. However, the 7-DPI serum was generally tested undiluted because of the relatively low antibody content and the slower diffusion of the 19S antibody.

Enzyme Reagents.—Trypsin (Nutritional Biochemicals Corp., Cleveland, Ohio), 3 × crystallized chymotrypsin (Worthington Biochemical Corp., Freehold, N. J.), diisopropyl-fluorophosphate (DFP)-chymotrypsin (Worthington) and 5 × crystalline soybean trypsin inhibitor (Nutritional Biochemicals) were diluted in phosphate-buffered saline (PBS) for virus treatment studies.

EXPERIMENTAL AND RESULTS

General Findings.—Preliminary observations demonstrated that a 7-DPI guinea pig serum pool gave two precipitin bands with a 140S-Hi-pass virus preparation while anti-140S serum gave only a single band (Fig. 1) as did hyperimmune serum. For reasons that will become evident later, the precipitin band closest to the 7-DPI serum well will be designated as the 140S-b band, and the one closest to the antigen well as the 140S-ab band. The antigenic particles responsible for these bands will be indicated accordingly. Acridine orange staining of such reactions demonstrated that both of the 7-DPI serum precipitin bands stained flame red which indicated the presence of single-stranded RNA-containing antigen (16).

Approximately 20 different 140S-Hi-pass preparations were examined and all gave the same reaction. An attempt was made to separate the two possible virus components by ultracentrifugation on CsCl gradients, but a single light-scattering zone was obtained which contained both components. From this

finding it may be assumed that the 140S-b and 140S-ab particles have essentially the same density of 1.43 g/ml previously determined for FMDV (19).

The possibility that the double banding was attributable to an aberrant 7-DPI serum pool was explored. Sera from 20 infected guinea pigs were collected 7-DPI and examined individually, and all gave two bands with 140S-Hi-pass preparations. An additional group of guinea pigs was infected and blood collected 3, 4, 5, 6, 7, 10, 12, 14, and 17 DPI and individual sera examined. None of the individual sera collected at 3 DPI gave any reaction while all of the 4-, 5-, 6-, and 7-DPI sera reacted and gave double bands. At 10 DPI, double bands were vaguely evident with sera from five of the eight remaining guinea pigs, but by 12 DPI, no clear evidence of double bands was obtained. With the

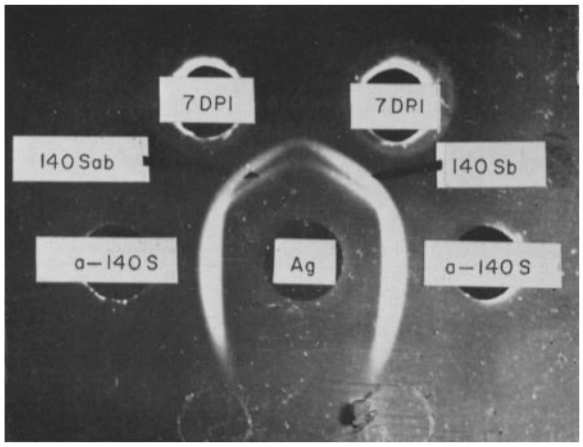

FIG. 1. Precipitin lines formed with 140S-Hi-pass FMDV (Ag) with undiluted 7-day post-infection guinea pig serum (7-DPI) and guinea pig anti-140S serum (a-140S) diluted ¼.

14- and 17-DPI samples, only a single precipitin band occurred. Thus, the loss of the double-banding effect corresponds to the time of appearance of significant levels of 7S antibody and the decline of 19S antibody (3).

To verify the nature of the antibody occurring in the different sera, 7 DPI, hyperimmune and anti-140S sera were ultracentrifuged on KBr-$NaNO_3$ density gradients and the 1.0-ml fractions collected from the tubes examined for antibody activity. The bottom fraction (fraction 1) of the 7-DPI serum contained most of the antibody activity and gave two precipitin bands; some activity was also evident in the second fraction. The third fractions of the hyperimmune and anti-140S sera were the most active and gave single bands; weak precipitin reactions were also obtained with fractions 2 and 4. Fraction 1 of the 7-DPI serum and fraction 3 of the hyperimmune and anti-140S sera were examined by immunoelectrophoresis and tested against both 140S-Hi-pass virus (Fig. 2) and

rabbit antiserum to guinea pig serum. Tested against the virus antigen, two precipitin arcs in the fast-γ-globulin region were obtained with fraction 1 of the 7-DPI serum, and a single band that extended from the slow- to fast-γ-globulin region with fraction 3 of the hyperimmune and anti-140S sera. With the latter two sera, the intensity of the precipitin arcs was much greater in the slow-γ-globulin region, and was barely evident in the fast-γ-globulin zone. The reaction with rabbit antiserum confirmed that fraction 1 of the 7-DPI serum contained primarily fast-γ- and α-globulin components, although traces of other

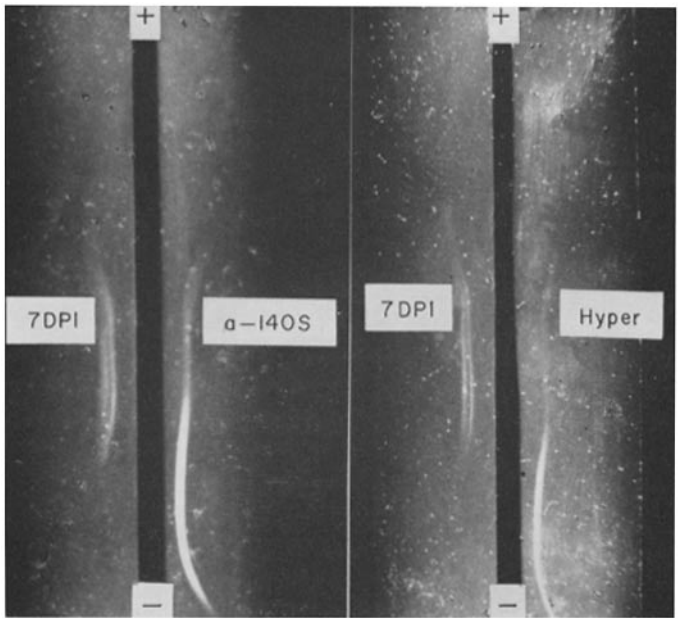

FIG. 2. Immunoelectrophoretic assay of ultracentrifugation, fraction 1 of 7-day postinfection guinea pig serum (7-DPI) and fraction 3 of anti-140S (a-140S) and hyperimmune (Hyper) sera tested against 140S-Hi-pass FMDV.

serum proteins were evident. It is clear that the antibody in the 7-DPI serum was a macroglobulin having fast-γ-globulin mobility (19Sγ_1), while the antibody in hyperimmune and anti-140S sera had a lower *s* rate and electrophoresed primarily as γ_2-globulin with trace amounts of γ_1-globulin.

Effect of Trypsin and Chymotrypsin on 140S-Hi-Pass Virus.—A recent report by Wild and Brown (20) indicated that the infectivity, density, and antigenic activity of FMDV were altered by treatment with trypsin. In view of the relatively extended exposure of virus to cellular enzymes during virus growth, the possibility was considered that the two bands observed were due to "normal" virus and enzyme-altered virus. Therefore, 1-mg amounts of 140S-Hi-

pass virus were treated with 100 μg of trypsin or chymotrypsin for 15 min at 37°C. The untreated and enzyme-treated preparations were then tested against 7-DPI and anti-140S sera (Fig. 3). Tested against 7-DPI serum, the usual two bands were obtained with untreated virus. Chymotrypsin-treated virus also gave two bands, but neither clearly coalesced with the untreated virus bands. Trypsin-treated virus gave a single band which did not coalesce with either of the untreated virus bands, and additional testing demonstrated that it did not coalesce precisely with either of the two chymotrypsin-treated virus bands. Thus, testing these preparations against 7-DPI serum indicated that a marked alteration of antigenic characteristics had occurred upon enzyme treatment. In contrast to the reactions obtained with 7-DPI serum, the anti-140S serum gave

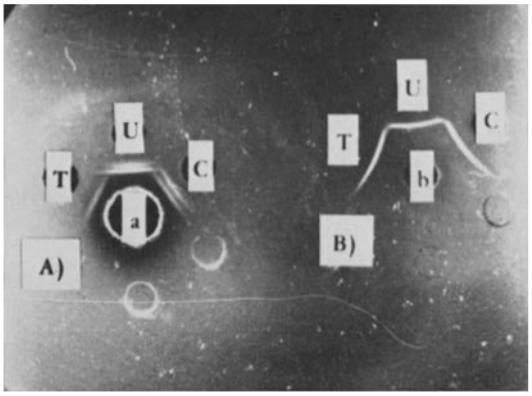

FIG. 3. Precipitin lines formed with untreated (U), trypsin-treated (T), and chymotrypsin-treated (C) 140S-Hi-pass FMDV with 7-day postinfection guinea pig serum (a) and anti-140S serum (b).

single coalescing bands indicating that no evident antigenic alteration had occurred. Hyperimmune serum reacted in the same manner. The effect of trypsin on virus was inhibited by soybean trypsin inhibitor and DFP-chymotrypsin had no effect on the virus.

An alteration in the surface characteristics of the enzyme-treated virus was also demonstrated by immunoelectrophoresis examination of these preparations (Fig. 4). The virus samples were electrophoresed and patterns developed with 7-DPI and anti-140S sera. Untreated virus remained essentially at the origin, chymotrypsin treatment increased the mobility somewhat, while trypsin treatment caused a marked increase in mobility. Reactions obtained with 7-DPI serum were weak, but two bands were evident with both untreated and chymotrypsin-treated virus although they are not readily seen in the figure presented. The increased mobility of chymotrypsin-treated virus agreed with the findings of Vande Woude et al. (11), but was contrary to their results with trypsin-

treated virus. However, reexamination of the effect of freshly prepared trypsin on virus confirmed the results reported here.[3]

Precipitin reactions obtained with untreated, trypsin- and chymotrypsin-treated virus, and 7-DPI serum were stained with acridine orange, and all of the bands took up the stain to give red fluorescence. Therefore, the bands were produced by RNA-containing antigens. The information sought in these experiments was not obtained; however, the results of experiments to be presented demonstrated that the double bands obtained with 140S-Hi-pass virus could not be attributed to enzymatic alteration of the virus particles.

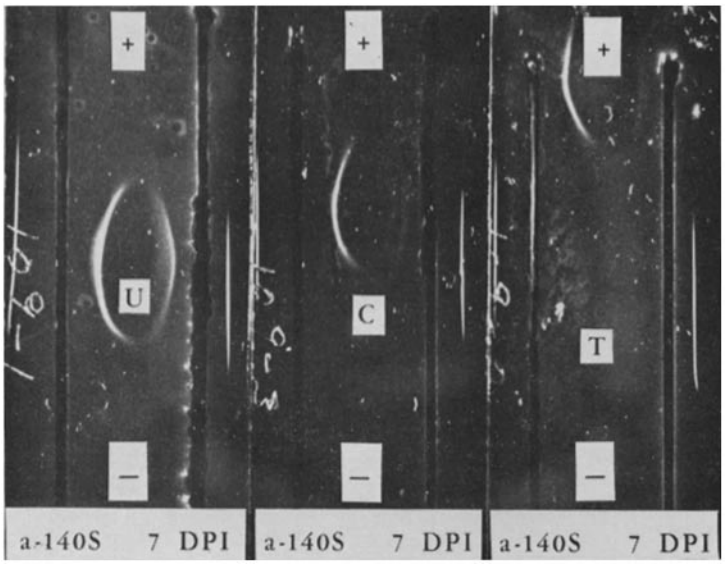

FIG. 4. Immunoelectrophoresis of FMDV, A-119, that was untreated (U), chymotrypsin-treated (C), and trypsin-treated (T), tested against 7-day postinfection serum (7-DPI) and specific anti-140S serum (a-140S) from guinea pigs.

In general, these observations indicated that modifications of the protein coat of FMDV could readily be detected with guinea pig antibody of the 19S class, while antibodies of the 7S class were unable to do so.

Absorption Studies and Purification of 140S-b Particles.—The fact that double bands occurred with 7-DPI serum and 140S-Hi-pass virus indicated that if the reaction was actually attributable to two antigenic particles, there would be a disproportion either in the amount of each or in the amount of antibodies directed against them or both. Therefore, an absorption experiment was performed to eliminate the possibility that the reaction was due to an artifact such

[3] Vande Woude, G. Personal communication.

as the Liesegang's phenomenon, to provide some information on the possible preparation of specific antigen or antiserum reagents, and to determine the effect of absorption on the neutralizing activity of the serum.

To 1.0-ml amounts of 7-DPI serum was added 25, 50, 100, 200, 400, and 800 μg of 140S-Hi-pass virus in a volume of 0.25 ml. Serum receiving buffer was also included as a control. The mixtures were incubated 1 hr at 37°C and then placed at 4°C for 4 days. The mixtures were shaken frequently during this incubation period. They were finally centrifuged for 1 hr at 2000 rpm in the cold and the supernatant fluids collected. The supernatant fluids were tested for antibody

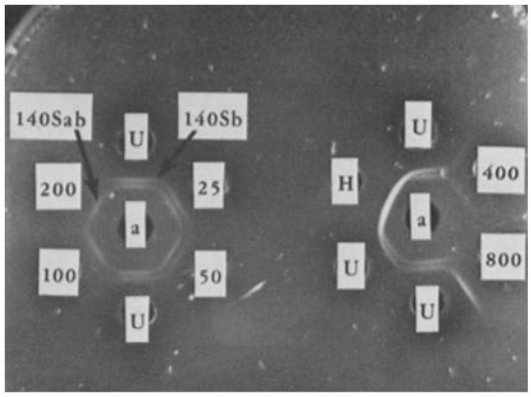

FIG. 5. Precipitin activity of 7-day postinfection guinea pig serum (7-DPI) absorbed with varying amounts of purified FMDV, A-119, tissue culture–high passage virus (140S-Hi-pass). The serum was absorbed with from 25 to 800 μg of 140S-Hi-pass virus/ml of serum, and the numbers in the figure indicate the dosage used. The absorbed serum was tested against 500 μg/ml of 140S-Hi-pass virus (a). Unabsorbed 7-DPI serum (U) and hyperimmune serum (H) were included for control purposes. The precipitin bands attributed to 140S-ab and 140S-b particles are indicated.

activity by agar gel analysis against 140S-Hi-pass virus (Fig. 5), and then treated with 0.05% acetylethylenimine (AEI) (21) for 24 hr at 37°C to inactivate any residual viable virus. The virus-inactivating activity of the AEI was then neutralized by adding sodium thiosulfate (21). The AEI-treated supernates were tested for virus-neutralizing activity in suckling mice (Table I).

Two precipitin bands were still obtained with 7-DPI serum treated with 25 and 50 μg of virus, but with 100 or 200 μg amounts, the 140S-b band was eliminated. With 400 and 800 μg of virus, the 140S-b component was in excess as evidenced by the precipitin band occurring between the control 7-DPI serum wells and these high-dose antigen-serum wells, and the coalescence of this band with the 140S-b band. The antibody responsible for the 140S-ab band was markedly reduced by absorption with 400 μg of virus and eliminated with 800

μg of virus. Thus, serum absorbed with 400 μg of virus contained free 140S-b particles, but also antibody capable of reacting with 140S-ab particles. These observations clearly indicated that two different antigenic components occurred in 140S-Hi-pass virus preparations, and that antibodies of two different specificities were present in 7-DPI serum.

With respect to the neutralizing activity of the absorbed serum (Table I), the addition of 200 μg of virus abolished the neutralizing activity but not its ability to form a precipitin band with 140S-ab particles (Fig. 5). This suggests that the residual antibody reacting with 140S-ab particles combines with an antigenic determinant that is not critical with respect to virus neutralization.

TABLE I

Neutralizing and Precipitating Activity of Supernatant Fluids of 7-Day Postinfection Guinea Pig Serum Absorbed with Varying Amounts of Purified, Tissue Culture–High Passage Foot-and-Mouth Disease Virus (140S-Hi-Pass)

Virus used* for absorption per ml serum	PD_{50}‡	Precipitating activity vs.	
		140S-ab	140S-b
μg			
0	2.22	+	+
25	1.52	+	+
50	1.35	+	+
100	0.91	+	−
200	<0.3	+	−
400	<0.3	±	−
800	<0.3	−	−

* Serum and virus were combined and incubated for 1 hr at 37°C followed by 4 days at 4°C. The supernatant fluids were collected by centrifugation and treated with 0.05% acetylethylenimine to inactivate any residual viable virus.

‡ Log_{10} of the reciprocal of the serum dilution protecting 5 of 10 suckling mice against 100 LD_{50} of virus.

Absorption experiments such as this demonstrated that 7-DPI serum absorbed with 150 μg of 140S-Hi-pass virus per milliliter of serum would provide an antiserum reagent specific for 140S-ab particles (anti–140S-ab). These experiments also suggested a method for purifying 140S-b particles; therefore, experiments to prepare specific anti–140S-ab serum and to purify 140S-b particles were carried out. 10 ml of 7-DPI serum was mixed with 1.5 mg of 140S-Hi-pass virus (150 μg virus/ml serum) and incubated with occasional mixing for 5 days at 4°C. The mixture was centrifuged at 4°C for 1 hr at 16,000 rpm and the supernatant fluid collected. Agar gel precipitin analysis demonstrated that this absorbed serum reacted only with 140S-ab particles (Fig. 7 *b*); it was also shown that it did not neutralize virus. A portion of this absorbed serum

was then combined with additional 140S-Hi-pass virus using 350 μg of virus per milliliter of absorbed serum. This dose of virus was selected so that a slight excess of antibody reactive with 140S-ab particles would occur in the supernatant fluid. This mixture was considered to consist of 140S-ab particles com-

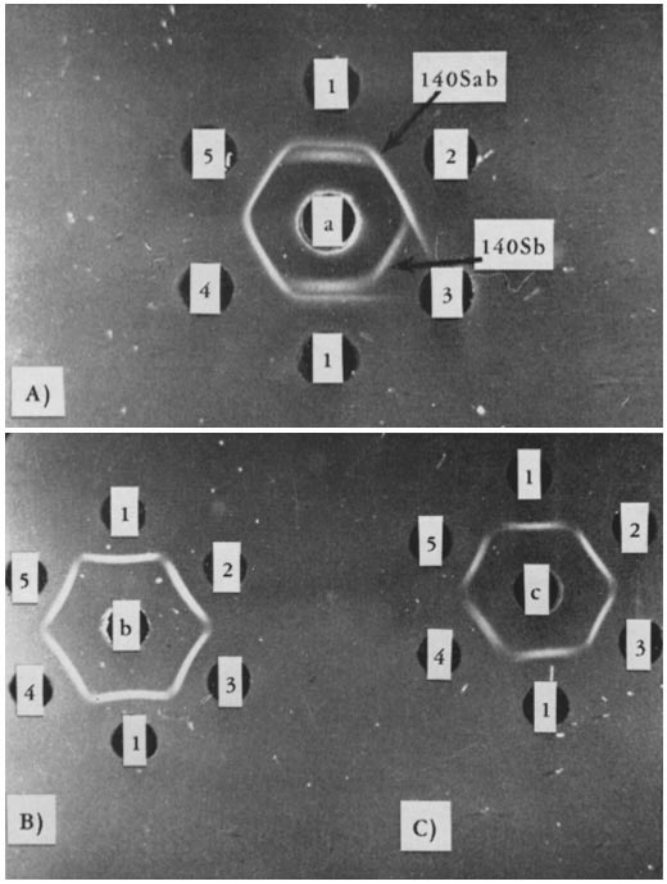

FIG. 6. Precipitin reactions of guinea pig (a) 7-day postinfection serum (undiluted); (b) anti-140S serum (diluted 1/5); and (c) hyperimmune serum (diluted 1/3) with FMDV, A-119, preparations. (1) 140S-Hi-pass virus; (2) 140S-Lo-pass virus; (3) 140S-b virus; (4) 140S-TC-GP virus; and (5) 140S-GP virus. All virus preparations were at approximately 500 μg/ml.

plexed with antibody (precipitate), a small amount of free antibody to 140S-ab particles, and free 140S-b particles. After 4 days of incubation at 4°C, the precipitate was removed by high speed centrifugation and the supernatant fluid collected. The 140S-b particles in the supernatant fluid were then isolated by ultracentrifugation into isodense CsCl in the manner used for virus purification.

A light-scattering band characteristic of FMDV was formed in the CsCl layer and was collected and dialyzed free of CsCl in the usual manner.

The precipitin reactions obtained with the 140S-b preparation, and the reactivity of the anti-140S-ab serum (7-DPI serum absorbed with 150 μg of 140 S-Hi-pass virus) are shown in Figs. 6 and 7 in conjunction with other samples.

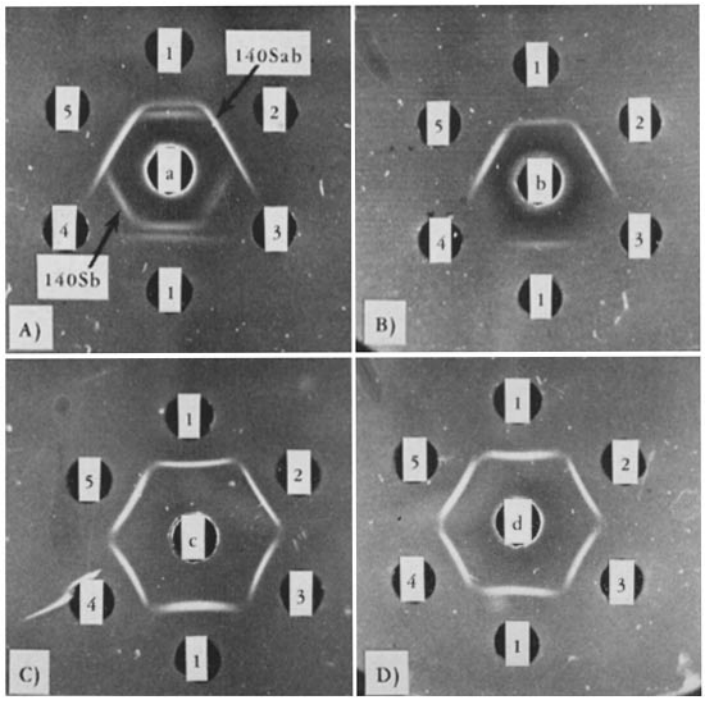

FIG. 7. Precipitin reactions of guinea pig (a) 7-day postinfection serum (undiluted); (b) 7-day postinfection serum absorbed with 150 μg of 140S-Hi-pass virus/ml serum (undiluted); (c) anti-140S serum (diluted 1/5); and (d) hyperimmune serum (diluted 1/3) with FMDV, A-119, preparations. (1) 140S-Hi-pass virus; (2) 140S-Lg.pl. virus; (3) 140S-Sm.pl. virus; (4) 140S-b virus; and (5) 140S-Lo-pass virus. All virus preparations were at approximately 500 μg/ml.

The 140S-b preparation reacted with 7-DPI serum and formed a band that coalesced with the 140S-b band of the 140S-Hi-pass preparation (Fig. 6 *a*). It did not react with the anti-140S-ab serum as indicated previously (Fig. 7 *b*). Tested against anti-140S sera (Fig. 6 *b*) and hyperimmune sera (Fig. 6 *c*), the 140S-b preparation gave a single band that coalesced with the single band obtained with the 140S-Hi-pass preparation and there was no indication of spurring. This again illustrated the marked difference in reactivity of antibodies in 7-DPI serum and those of hyperimmune or anti-140S sera.

Reactivity of Alternate Sources of FMDV, A-119.—The experiments described up to this point involved only 140S-Hi-pass virus. We examined the reactivity of low passage tissue culture virus (140S-Lo-pass), a guinea pig vesicular fluid source of virus (140S-GP), 140S-Hi-pass virus that had been adapted to guinea pigs (140S-TC-GP), and the large and small plaque variants (140S-Lg.pl., and 140S-Sm.pl., respectively) of virus derived from the 140S-Hi-pass virus. The large and small plaque variants were of particular interest as it was demonstrated by neutralization studies that the two isolates were antigenically different, although they were related.[4]

The initial experiments compared the reactivity of 140S-Hi-pass, 140S-b, 140S-Lo-pass, and the guinea pig viruses when tested against 7-DPI (Fig. 6 *a*), anti-140S (Fig. 6 *b*), and hyperimmune (Fig. 6 *c*) sera. The 7-DPI serum gave the usual two bands with the 140S-Hi-pass virus, and the 140S-b antigen coalesced with the 140S-b band of the 140S-Hi-pass virus preparation; however, the 140S-Lo-pass, 140S-GP, and 140S-TC-GP preparations gave only a single band that coalesced with the 140S-ab band of the 140S-Hi-pass preparation. Thus, the low-passage tissue culture virus and the guinea pig adapted viruses appeared to be devoid of 140S-b particles, or at least these particles were not demonstrable by this assay. It should be noted that the precipitin band formed by 140S-b particles neither crossed nor fused with the 140S-ab band. The possible significance of this reaction will be presented in the Discussion.

As in the case of the enzyme-treated virus preparations, the anti-140S (Fig. 6 *b*) and hyperimmune (Fig. 6 *c*) sera gave single coalescing precipitin bands with the different antigen preparations and provided no evidence of antigenic differences between the various preparations.

The 140S-Lg.pl. and 140S-Sm.pl. preparations were next examined along with other virus preparations (Fig. 7). The 140S-Lg.pl. virus consisted of only demonstrable 140S-ab particles while the 140S-Sm.pl. preparation appeared to correspond with 140S-b particles. It may be noted, however, that the 140S-Sm. pl. band did not coalesce precisely with the 140S-b band of the 140S-Hi-pass preparation. This indicated that the 140S-b preparation was different than the 140S-Sm.pl. virus; therefore, a plaque assay was performed with the 140S-b preparation on calf kidney cell cultures, and plaques having a diameter of 4–6 mm were evident at 48 hr. Bottles at the end of the virus dilution series contained well-isolated plaques, six of which were picked and then passaged twice in calf kidney cell cultures. Moderate volumes of each of the six plaque isolates were grown, concentrated with $(NH_4)_2SO_4$ and checked for antigen activity. All six produced a precipitin band that coalesced precisely with the 140S-b preparation. One of these plaque isolates came from a bottle in which only a single plaque occurred and its was selected for further virus production. Virus isolated from this plaque was grown in BHK-21 culture and purified as de-

[4] Martinsen, J. Personal communication.

scribed previously. Agar gel analysis demonstrated that this 140S-b preparation coalesced with the 140S-b band of the 140S-Hi-pass preparation and with the original 140S-b preparation, but produced a spur with the 140S-Sm.pl. virus. The failure to obtain a spur with the plaque isolated 140S-b particles and the 140S-b component of the 140S-Hi-pass virus would suggest that the 140S-Sm.pl. component was a minor constituent of 140S-Hi-pass preparations and had relatively little influence on the precipitin reactions obtained with the latter samples. Again, the anti-140S and hyperimmune sera were unable to distinguish between the different antigenic variants of FMDV, A-119 (Figs. 7 *c* and 7 *d*).

DISCUSSION

The findings presented here raise two major questions: (*a*) what is the significance and nature of the antigenically different virus particles occurring in various preparations of what must be presumed to be a single serological type and strain of FMDV; and (*b*) why can 7-DPI serum distinguish between these antigenic variants while hyperimmune and anti-140S sera are unable to do so? This discussion will be concerned only with the first question. However, some brief comment concerning the nature of the antibody occurring in the different sera should be made.

Density gradient ultracentrifugation and immunoelectrophoresis analysis (Fig. 2) of the 7-DPI serum demonstrated that the antibodies responsible for the double bands formed with 140S-Hi-pass virus were both of the 19S class. Thus, the double banding observed could not be attributed to two different physical-chemical classes of antibodies having different specificities. These studies also demonstrated that the antibodies occurring in the hyperimmune and anti-140S sera were of the low *s* rate class, and were of γ_2- and γ_1-globulin mobility, i.e., 7Sγ_2 and 7Sγ_1. One could assume, therefore, that the differing reactivities observed with these sera were due to differences in the specificity of 19S and 7S antibodies.

In an attempt to explain the precipitin patterns obtained with 7-DPI serum during this study, the interpretations developed by Ouchterlony (22, 23) were used. It should be recognized, however, that numerous assumptions have been made and, at best, the following explanation is merely meant to provide a hypothesis for subsequent testing. Fig. 8 provides a composite diagrammatic representation of the reactions obtained with 7-DPI serum tested against 140S-Hi-pass virus, 140S-b virus, and 140S-Lo-pass virus. The various reactants may be tentatively characterized as follows:

(*a*) 7-DPI serum contains at least two different antibodies having distinct specificities, i.e., anti–a-determinant (A) and anti–b-determinant (B) where A is at higher concentration than B.

(*b*) 140S-Hi-pass virus contains at least three different antigenic variants of virus. One of the variants possesses both a- and b-determinant sites (140S-ab),

another only b-determinants (140S-b) and the third a b-related determinant (140S-Sm.pl.). The 140S-ab particles are the major constituent in 140S-Lg.pl., 140S-Lo-pass, 140S-GP and 140S-TC-GP preparations, whereas 140S-b particles appear to be the major component in 140S-Hi-pass preparations. The 140S-Sm.pl. virus is devoid of the a-determinant sites and is antigenically related to 140S-b particles.

A factor that aided in this interpretation of these reactions was the failure of the 140S-b band to either cross or fuse with the 140S-ab band (Figs. 6 and 7). This would be equivalent to reaction Type IV described by Ouchterlony which is attributed to two antigenic particles having a common antigenic determinant (b-determinant), but one of them having an additional distinct one (a-deter-

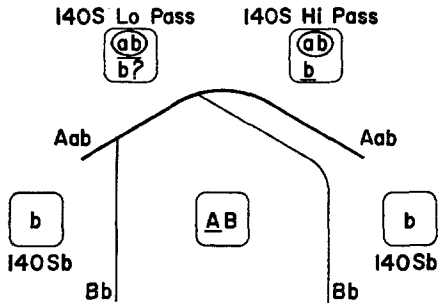

FIG. 8. Diagrammatic representation of precipitin reactions obtained with 7-day postinfection guinea pig serum (center well) and FMDV, A-119, preparations: 140S-Hi-pass, 140S-Lo-pass, and 140S-b. Letters a and b indicate unrelated antigenic sites and ab inside ellipse indicates an antigenic particle on which both determinants occur. A and B are the corresponding antibodies to these antigenic determinants. Reactants underlined are at a relatively higher concentration.

minant) (23). It is for this reason the viruses have been termed 140S-ab and 140S-b particles.

In Fig. 8, the component assumed to be in highest concentration in a given reservoir is underlined; however, quantitative assays for the individual reactants have not been developed. The importance of relative concentration of reactants has been stressed by Ouchterlony (23) and may be appreciated by considering Fig. 8. If, for example, 140S-Hi-pass preparations were predominantly multideterminant ab-type particles, the Aab band would establish an immune barrier that B could not penetrate, and the only possibility of obtaining a second band would be that b-determinant particles were in sufficient concentration to form a Bb band on the serum well side of the Aab band. Considering the relatively large size of virus particles and their resultant slow diffusion rate, it is likely that the faster diffusing B antibody would reach the Aab band area before the 140S-b particles, thus eliminating the possibility of Bb band

formation. This situation may exist with 140S-Lo-pass virus and suggests why it is difficult to demonstrate 140S-b in such preparations. It is for this reason that the 140S-b component of the 140S-Lo-pass virus was assigned a question mark in Fig. 8.

With this interpretation of the results, the antigenic structure of "normal" or wild virus is probably one where both a- and b-determinants occur on the same particle. This is indicated by the fact that the guinea pig virus was of the 140S-ab type, and also that 140S-Hi-pass virus reverted to a predominant 140S-ab type upon passage in guinea pigs. Apparently, 140S-b particles developed to predominance upon high tissue culture passage but, unfortunately, samples were not available of the various passage levels. Therefore, it was not possible to determine if the apparent shift from 140S-ab dominance to 140S-b dominance was a sudden or gradual process.

The interpretation of precipitin patterns obtained with 7-DPI serum does not consider the observations made with trypsin- and chymotrypsin-treated virus preparations nor with the 140S-Sm.pl. virus. Obviously, the interpretation is a gross simplification of the actual situation. Whether the antigenic differences observed with enzyme-treated virus are attributable to the exposure of masked antigenic determinants or to conformational changes occurring on the virus surface is not known. However, it is of interest that the 7-DPI serum was able to reveal these alterations indicating that the infected animals had been exposed and responded to these antigenic determinants during the course of the infection.

The finding that anti-a antibody was unable to neutralize the virus suggests that virus particles having the relatively simple structure and chemical composition of FMDV may contain both critical and noncritical neutralization sites. The occurrence of the a-determinants on "animal" virus, compared to highly tissue culture–adapted virus provides ample grounds for speculation as to its possible significance on the pathogenicity of FMDV; however, such speculation would be premature. Still this demonstration of an apparently noncritical antigenic determinant site in FMDV could reasonably be of concern in resolving problems related to the nonneutralizable fraction occurring in virus-antibody mixtures. The possibility that antibody combined to a noncritical antigenic site could sterically hinder the attachment of antibody to the critical site might be considered in view of analogous situations reported for various enzyme–anti-enzyme systems (24).

A point of interest will be the determination of the relative content of b-determinant sites on 140S-ab and 140S-b particles. If, as the results suggest, neutralization occurs through the b-determinant site, this site would also be expected to be responsible for the stimulation of neutralizing antibodies. If differences in b-determinant content were found with the two types of particles, a concomitant difference in immunizing ability might be anticipated. Thus,

for purposes of standardizing vaccines by in vitro assays, it may not suffice to calibrate to equivalent virus mass concentrations as reported by Graves et al. (25), but it would probably be necessary to calibrate to equivalent b-determinant content. Experiments are in progress to evaluate these possibilities.

Foot-and-mouth disease virus is a relatively simple virus consisting of a single-stranded RNA core and a protein coat. The 140S-Hi-pass virus used in these studies has been examined rigorously and reported to be homogeneous by chemical, ultracentrifugal, and electrophoretic tests (8, 10, 11) and to be composed of repeating units of a single polypeptide (26). Similarly, immunodiffusion assays utilizing antisera containing antibodies of the 7S class would also indicate homogeneity (14). However, immunological studies suggested density heterogeneity (13), and the studies of Martinsen[4] demonstrated plaque heterogeneity, which was related to antigenic differences between the plaque isolates. The present studies dramatically demonstrated antigenic heterogeneity and indicate that antibodies of the 19S class are far superior to antibodies of the 7S class in their discriminating capabilities.

Future studies on virus will necessitate detailed immunological and biological investigations to relate antigenic structure of these variants to their pathogenicity and immunogenicity. Similarly, physical and chemical studies will be required to determine if the antigenic differences are attributable to conformational differences of the protein coat at a tertiary or quaternary structural level, to amino acid content or sequence differences or both, or to the variable occurrence of additional protein constituent(s).

SUMMARY

Three antigenic variants of foot-and-mouth disease virus, type A, strain 119, were demonstrated in Ouchterlony analyses utilizing serum collected from guinea pigs 7 days postinfection (DPI). Such antisera contain antibodies of the 19S class. Guinea pig antisera that contained antibodies of the 7S class were unable to distinguish between the antigenic variants. Similarly, 19S antibody was able to demonstrate antigenic differences in trypsin- and chymotrypsin-treated viruses that were not detected by 7S antibody-containing antisera.

One of the antigenic variants of virus is apparently the wild type and is tentatively considered to have two antigenic determinant groupings termed the a- and b-sites (140S-ab). The 140S-ab variant was the sole or predominant antigenic type produced in guinea pigs and in large plaque-forming– and tissue culture–low passage sources of the virus. Another antigenic variant appears to possess only the b-site (140S-b) and was the major constituent in tissue culture–high passage virus preparations. The third variant, a small plaque former, was also devoid of the a-site and contains an antigenic determinant that is related

to, but not identical with, the b-site. This variant appears to be a minor constituent of tissue culture–high passage virus.

7-DPI serum could be absorbed with a suitable concentration of tissue culture–high passage virus to remove antibody reactive with the b-determinant site. This absorbed serum still precipitated 140S-ab virus by virtue of still containing antibody reactive with the a-determinant site; however, the neutralizing activity was eliminated. This suggests that the b-site is critical with respect to neutralization while the a-site is noncritical.

I wish to thank Mr. Jay Card for his excellent technical assistance, and Dr. John Graves, Mrs. Ethel Appelt, and Mr. Freddie Lawson for growing the various tissue culture sources of virus used in these studies.

BIBLIOGRAPHY

1. Cowan, K. M. 1968. Antigenic heterogeneity of foot-and-mouth disease virus as demonstrated with 7 days postinfection guinea pig serum by Ouchterlony analyses. *Fed. Proc.* **27:** 733.
2. Brown, F., B. Cartwright, and J. Newman. 1964. Further studies on the early antibody in the sera of cattle and guinea pigs infected with foot-and-mouth disease virus. *J. Immunol.* **93:** 397.
3. Cowan, K. M., and R. Trautman. 1965. Antibodies produced by guinea pigs infected with foot-and-mouth disease virus. *J. Immunol.* **94:** 858.
4. Cowan, K. M. 1966. Heterogeneity of antibodies produced by cattle infected with foot-and-mouth disease virus. *Amer. J. Vet. Res.* **27:** 1217.
5. McKercher, P. D., and A. R. Giordano. 1967 Foot-and-mouth disease in swine. II. Some physical-chemical characteristics of antibodies produced by chemically-treated and nontreated foot-and-mouth disease virus. *Arch. Gesamte Virusforsch.* **20:** 54.
6. Benacerraf, B., Z. Ovary, K. Bloch, and E. Franklin. 1963. Properties of guinea pig 7S antibodies. I. Electrophoretic separation of two types of guinea pig 7S antibodies. *J. Exp. Med.* **117:** 937.
7. MacPherson, I., and M. Stoker. 1962. Polyoma transformation of hamster cell clones—an investigation of genetic factors affecting cell competence. *Virology.* **16:** 147.
8. Bachrach, H. L., R. Trautman, and S. S. Breese, Jr. 1964. Chemical and physical properties of virtually pure foot-and-mouth disease virus. *Amer. J. Vet. Res.* **25:** 333.
9. Polatnick, J., and H. L. Bachrach. 1964. Production and purification of milligram amounts of foot-and-mouth disease virus from baby hamster kidney cell cultures. *Appl. Microbiol.* **12:** 368.
10. Matheka, H. D., H. L. Bachrach, and R. Trautman. 1966. Highly purified foot-and-mouth disease virus: Optical and biological measurements during zone electrophoresis in a glucose density gradient. *Z. Naturforsch.* **21b:** 774.
11. Vande Woude, G., R. Trautman, and H. L. Bachrach. 1967. The influence of the host cell, ionic strength and chymotrypsin on foot-and-mouth disease virus electrophoretic mobility. *Arch. Gesamte Virusforsch.* **20:** 71.

12. Breese, S. S., Jr., R. Trautman, and H. L. Bachrach. 1960. Analysis by electron microscopy and infectivity of foot-and-mouth disease virus in moving-boundary and zone ultracentrifugation. *Arch. Biochem. Biophys.* **87:** 1.
13. Cowan, K. M. 1966. Effect of Merthiolate on agar gel diffusion precipitin reactions with foot-and-mouth disease virus. *J. Immunol.* **97:** 647.
14. Cowan, K. M., and J. H. Graves. 1966. A third antigenic component associated with foot-and-mouth disease infection. *Virology.* **30:** 528.
15. Cowan, K. M. 1968. Immunochemical studies of foot-and-mouth disease. IV. Preparation and evaluation of antisera specific for virus, virus protein subunit and infection-associated antigen. *J. Immunol.* **101:** In press.
16. Cowan, K. M., and J. H. Graves. 1968. Immunochemical studies of foot-and-mouth disease. III. Acridine orange staining of agar gel precipitin reactions. *Virology.* **34:** 544.
17. Scheidegger, J. J. 1955. Une micro-méthode de l'immuno-électrophorése. *Int. Arch. Allergy Appl. Immunol.* **7:** 103.
18. Cowan, K. M., and R. Trautman. 1967. Immunochemical studies of foot-and-mouth disease. I. Complement fixation reactions with isolated antigenic components. *J. Immunol.* **99:** 729.
19. Trautman, R., and S. S. Breese, Jr. 1962. Isodensity ultracentrifugation of foot-and-mouth disease virus in caesium chloride. *J. Gen. Microbiol.* **27:** 231.
20. Wild, T., and F. Brown. 1967. Nature of the inactivating action of trypsin on foot-and-mouth disease virus. *J. Gen. Virol.* **1:** 247.
21. Brown, F., N. Hyslop, J. Crick, and A. Morrow. 1963. The use of Acetyethyleneimine in the production of inactivated foot-and-mouth disease vacine. *J. Hyg.* **61:** 337.
22. Ouchterlony, O. 1960. Interpretation of comparative immune precipitation patterns obtained by diffusion-in-gel techniques. *In* Immunochemical Approaches to Problems in Microbiology. M. Heidelberger and O. Plescia, editors. Rutgers University Press, New Brunswick. 5.
23. Ouchterlony, O. 1964. Gel-diffusion techniques. *In* Immunological Methods. J. Ackroyd, editor. F. A. Davis Co., Philadelphia. 55.
24. Cinader, B. 1967. Antibodies to enzymes—a discussion of the mechanism of inhibition and activation. *In* Antibodies to Biologically Active Molecules. B. Cinader, editor. Pergamon Press, Oxford. 85.
25. Graves, J. H., P. D. McKercher, H. E. Farris, Jr., and K. M. Cowan. 1968. Early response of cattle and swine to inactivated foot-and-mouth disease vaccine. *Res. Vet. Sci.* **9:** 35.
26. Vande Woude, G. F., and H. L. Bachrach. 1968. Evidence for a single structural polypeptide in foot-and-mouth disease virus. *Arch. Gesamte Virusforsch.* **23:** 353.