The Antibody Response in Pigs Inoculated with Attenuated African Swine Fever Virus*

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SUMMARY

Pigs were inoculated with a modified isolate of African swine fever virus (ASFV). Complement-fixing (CF) and agar gel diffusion precipitin (AGDP) antibodies could be detected in the serums of most pigs from 14-days post-inoculation (DPI) until their immunity was challenged with virulent ASFV at 117 DPI.

Reductive cleavage with 2-mercaptoethanol showed that serums collected at 14 to 35 DPI contained 19S antibody, but that the 7S antibody was dominant at 35 and 117 DPI. This distribution of antibody was confirmed by sucrose-gradient centrifugation. Nearly all of the early serums also contained 7S antibodies which fixed complement and reacted in the AGDP test. Pigs whose serums contained both CF and AGDP antibodies at time of challenge failed to develop acute disease while pigs without CF antibodies were usually not protected.

Pigs surviving challenge with virulent virus showed no increase in antibody titers, or reversion to 19S antibody.

INTRODUCTION

Studies on the development and characterization of antibodies in the serums of pigs surviving infection with African swine fever (ASF) have been severely limited because of the relatively few survivors.

Most pigs inoculated with modified virus vaccines (15,10,1) developed specific antibodies to African swine fever virus (ASFV) as determined by the complement fixation (CF) and agar gel diffusion precipitin (AGDP) tests. However, there are no reports that describe the time-course appearance of these antibodies and their physico-chemical properties following inoculation with one of the modified viruses and subsequent challenge with virulent virus. Therefore, one objective was to follow for four months the appearance and duration of the CF and AGDP response in the serums of pigs inoculated with a cell-culture-modified ASFV and after challenge with homologous but virulent virus. A second objective was to determine if antibodies followed the usual pattern in appearance of 7S and 19S velocity classes, loss of antigen-combining properties after reductive cleavage of the disulfide bonds, and possible relationship of these antibodies to the serologic reactions observed. The response to challenge was established by the presence or absence of severe clinical signs of acute disease within 30 days postchallenge (DPC). Absence of marked clinical signs during 30 days postchallenge was considered as evidence of a degree of refractivity.

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MATERIALS AND METHODS

VIRUSES

The $Lisbon_{60}$ isolate of ASFV, as modified by Ribeiro *et al* (15) and two further passages in this laboratory, was used to immunize the pigs. Each of 22 pigs was inoculated intramuscularly (IM) with 1.0 ml of ASFV-infected cell-culture fluids containing approximately 10^6 tissue culture infectious doses 50 percent of virus.

Virulent virus for the challenge inoculation was prepared as a 10 percent suspension of spleen taken from pigs that died from ASFV seven to nine days after inoculation with the virulent $Lisbon_{60}$ isolate. The challenge inoculum was diluted in minimal Eagle's medium[1] to contain 10^4 pig lethal doses of ASFV and was administered (IM).

Viral antigens for use in the AGDP and CF tests were prepared from ASFV-in-

1. Baltimore Biological Laboratory, Baltimore, Maryland.

fected pig kidney (PK_{13}) cells as described by Hess *et al* (10). Uninfected cultures were treated in the same way to be used as controls.

Virus Isolation

The presence of virus in the blood and tissues of the pigs was determined by the hemadsorption (HA) test in pig leukocyte cultures (11). Lungs, spleens, and lymph nodes from each pig were pooled and prepared as 10 percent suspensions in phosphate buffered saline (PBS) for inoculation into leukocyte cultures. In addition, two pigs were each inoculated with 5.0 ml of the same tissue suspension to determine the possible presence of virulent virus.

Pigs

Purebred Tamworth pigs, 2½ months old, were used. Because of fighting, four of the 22 pigs inoculated with the modified ASFV were killed before challenge at 117 days postinoculation (DPI). Temperatures of all pigs were taken daily. All pigs that died following challenge inoculation, as well as the survivors that were destroyed at 23 DPC, were necropsied to determine the presence or absence of ASF lesions.

Agar Gel Diffusion Precipitin Tests

Precipitating antibody was assayed using 1.5 percent agar in pH 8.6 borate buffer (16). Two-fold dilutions of the serums were made in buffer and tests were controlled using preinoculation serums and uninfected PK_{13} cells as antigen.

Complement Fixation Tests

Complement fixing antibody was determined as outlined by Cowan (4) and five 50 percent hemolytic units of complement (5-C' H_{50}) were used with overnight fixation. The highest dilution of serum showing 50 percent or less hemolysis was considered to be the endpoint.

Ultracentrifugation Studies

Three serums were selected for assay of 7S and 19S antibodies following ultracentrifugation. Two of these serums were selected because of the persistence of significant titer and one because of early appearance and rapid decline of antibody. The 7S and 19S velocity classes of antibodies were separated by the method of Edelman *et al* (8). Serums were diluted 1:2 in PBS and 0.5 ml overlaid, with some mixing to prevent droplet formation, onto a sucrose-gradient column. The gradient was prepared by placing 1.0 ml of 40 percent, 2.0 ml of 25 percent, and 1.0 ml of 10 percent sucrose in PBS in a centrifuge tube. The layers were allowed to diffuse for four hours at 5°C before the serum sample was layered on the top. All samples were centrifuged at 39,000 RPM with the brake off in a Spinco[1] SW-39 rotor for 18 hours at approximately 21°C. After centrifugation, the top 3.5 ml containing a brown band located one-third of the distance down the tube was carefully removed with a syringe. The bottom 1.0 ml was mixed with a pellet, formed at the base of the tube, and removed. An additional 2.5 ml of buffer, in 0.5 ml amounts, was used to rinse the bottom. The upper layer (called 7S) and the lower 19S layer, and rinse were dialyzed against 1,000 volumes of saline to remove the sucrose. They were then further dialyzed against a 1:5 dilution of PBS, lyophilized, and redissolved in sufficient double-distilled water to make a final volume of 0.5 ml, equivalent to a 1:2 dilution of the original serum.

Treatment with 2-Mercaptoethanol

Serums from 12 of 22 pigs were assayed for antibody after treatment with 2-mercaptoethanol (2-Me). The duration and type of antibody following inoculation with modified virus and subsequent to challenge were the criteria used in selecting serums from the 12 pigs for treatment with 2-Me. The procedure was described by Osler *et al* (14) and the treated serums were tested for antibody by the AGDP test.

RESULTS

Nearly all of the 22 pigs inoculated with attenuated $Lisbon_{60}$ ASFV had a thermal response at 3 to 5 DPI. The temperatures of the pigs for the remainder of the 117-DPI period were in the normal range except for No 406, which had a one-day temperature rise to 107.4 F at 61 DPI, and No 405, which had a slight temperature rise to 104.4 F at 95 and 106 DPI.

On challenge inoculation at 117 DPI, the temperatures of all pigs were in the normal range, and they exhibited no clinical signs of ASF. Of the 18 pigs whose immunity

1. Beckman Instruments, Palo Alto, California.

TABLE I. Antibody Response in Pigs as Assayed by Agar Gel Diffusion Precipitin (AGDP) and Complement Fixation (CF) Tests Following Inoculation with Modified African Swine Fever Virus and Challenge Inoculation.

Pig no.	Postinoculation																				Postchallenge			
	0 days		7 days		14 days		20 days		35 days		49 days		64 days		78 days		92 days		117 days**		14 days		23 days	
	AGPD*	CF*	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF
09	0	0	0	0	0	20	8	20	8	40	16	40	4	20	8	20	4	40	8	80	2	10	4	0
80	0	0	0	0	2	20	16	20	8	80	8	20	4	10	8	20	4	20	4	40	4	10	4	10
256D	0	0	0	0	0	0	1	0	2	0	4	0	1	0	0	0	Dead							
259D	0	0	0	0	2	10	4	0	4	0	8	0	2	0	2	10	1	0	0	0	Dead			
260	0	0	0	0	2	10	4	40	4	40	8	40	8	80	16	160	16	160	4	40	4	20	4	0
266D	0	0	0	0	0	0	2	0	8	40	0	0	0	0	1	0	1	0	0	0	Dead			
267	0	0	0	20	1	80	4	20	1	10	8	80	4	80	4	40	Dead							
271	0	0	0	0	0	10	2	0	2	0	2	0	2	10	1	0	4	160	2	80	2	20	2	0
272	0	0	0	0	0	20	4	10	4	20	4	10	4	10	1	10	1	10	2	0	32	320	Dead	
273	0	0	0	0	4	40	4	80	8	40	2	40	4	1	4	40	Dead							
276	0	0	0	0	0	0	2	10	4	10	1	10	1	10	0	20	1	0	1	0	Dead			
277	0	0	0	0	1	20	8	80	8	40	4	20	8	40	8	20	1	20	2	40	4	40	4	10
281	0	0	0	0	16	40	16	40	4	20	4	10	8	20	4	20	4	20	2	80	1	10	2	0
282	0	0	0	0	4	20	4	20	4	20	2	10	1	10	1	10	2	10	1	40	1	10	1	0
283	0	0	0	0	4	40	8	40	4	20	4	20	Dead											
284	0	0	0	0	0	10	0	0	0	0	0	0	NT	NT	0	0	0	0	0	20	4	20	2	0
285	0	0	0	0	1	10	4	10	8	40	4	20	4	20	4	10	2	10	8	40	4	80	8	0
286	0	0	0	20	0	20	2	10	8	20	8	10	4	10	4	10	4	20	4	40	2	20	2	10
402	0	0	0	20	1	20	2	10	2	10	2	10	0	20	0	10	0	0	1	0	4	10	8	0
405	0	0	0	0	2	20	4	40	8	80	8	20	8	40	4	20	4	40	4	80	2	20	4	0
406	0	0	0	0	2	10	8	10	8	10	4	10	64	640	64	640	64	640	64	320	16	160	8	20
410	0	0	0	0	1	0	1	0	1	0	2	0	1	10	8	10	2	80	4	80	2	20	2	0

*Reciprocal of dilution. **Challenge. NT = not tested.

was challenged with the virulent $Lisbon_{60}$ ASFV, all but one (No 284) had a thermal response, and 4 pigs died from ASF. Two pigs died at 7 DPC, the 3rd at 9 DPC, and the 4th at 22 DPC. A pool of lung and spleen was prepared as a 10 percent suspension from each pig killed at 23 DPC. A composite of the tissue suspension from each of the 14 pigs was inoculated into each of two pigs. One inoculated pig died from ASF at 18 DPI. A 10 percent suspension of spleen from this animal was inoculated into a 3rd pig. This pig died from ASFV at 9 DPI. The 2nd pig inoculated with the pooled tissue suspension had no clinical signs of ASF but died after challenge inoculation.

Antibody was detected by the CF test in serums of 3 of 22 pigs on 7 DPI. At 14 DPI, the AGDP test also gave positive results with 14 of 18 serums (Table I). Antibodies persisted in the subsequent serum samples, both before and after challenge inoculation.

The CF titers declined in serum samples taken at 20 DPI. Only 16 of 22 were positive for CF antibody.

The difference in degree of precipitating and CF reactions continued in subsequent bi-weekly bleedings, with an average of 90 percent of the serums giving a positive reaction with the AGDP test, as compared with an average of 80 percent with the CF test. At 117 DPI with attenuated $Lisbon_{60}$ virus, 83 and 72 percent of 18 pigs had AGDP or CF antibodies, respectively. Of these pigs, 12 had both CF and AGDP antibodies.

Each of 18 pigs was given challenge inoculation at 117 DPI with virulent $Lisbon_{60}$ virus. Both CF and AGDP antibodies were detected at 14 DPC; precipitin reaction persisted, but only 4 of 14 reactions with the CF test persisted through 23 DPC (Table I).

The data in Table II show that the AGDP antibody in the early serums prepared at 14 and 20 DPI are sensitive to reductive cleavage by 2-Me with 4 of 7 and 9 of 11 serums, respectively, showing a decrease in AGDP titers following reaction with 2-Me. The AGDP titers were reduced by 2-Me at 35 DPI. The serum from only one pig (405) had 2-Me sensitive antibody at 64 and 92 DPI.

Serums of pigs 259 and 272 were also negative following treatment on the 92nd day. The significance of the effect of 2-Me was questionable as the titer of the serum before treatment was only 1:2.

Serums from 3 pigs (Nos 260, 281, and 406) bled at 14, 20, 35, 49, and 64 DPI with attenuated $Lisbon_{60}$ virus and at 14 DPC, were examined for distribution of the 7S and 19S antibodies (Table III). In the early serums, 14 to 35 DPI, 7 of the 9 samples had CF antibody in the 7S zone. There was no change in the location after centrifugation of the CF activity in the other serums taken after 35 DPI or in the post-

TABLE II. The Effect of 2-Mercaptoethanol on the Antigen Binding Capacity as Determined by the Agar Gel Diffusion Precipitin Test in the Serums of Pigs Following Inoculation with Lisbon Virus and Virulent Challenge

Ear tag no.	Days postinoculation														Days postchallenge			
	7		14		20		35		64		92		117		14		23	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
09	–	–	–	–	8+	4	4	4	4	4	4	4	4	4	2	2	2	2
259	–	–	4	2	4	2	4	4	4	4	2	–	–	–	Dead			
260	–	–	4	4	8	8	8	4	4	4	8	8	8	8	4	4	4	4
266	–	–	–	–	4	2	4	4	2	2	2	2	–	–	Dead			
271	–	–	–	–	4	2	2	2	2	2	2	2	4	4	2	2	2	2
272	–	–	–	–	8	2	4	4	4	4	2	–	–	–	32	32	Dead	
281	–	–	8	8	8	8	8	4	2	2	2	2	2	2	2	2	2	2
284	–	–	–	–	–	–	–	–	–	–	–	–	–	–	4	4	2	2
402	–	–	2	2	4	2	2	2	–	–	–	–	–	–	4	4	4	4
405	–	–	4	2	8	4	4	4	8	4	4	2	4	4	4	4	2	2
406	–	–	2	–	4	–	4	4	64	64	64	64	64	64	16	16	8	8
410	–	–	2	–	2	–	2	2	–	–	2	2	2	–	2	2	2	2

Pre = before treatment with 2-Mercaptoethanol.
Post = after treatment with 2-Mercaptoethanol.
\+ = reciprocal titers highest dilution showing visible line.
– = negative at initial dilution of 1:2.

TABLE III. Distribution of 7S and 19S Antibody in the Serum of Lisbon-Vaccinated Pigs as Assayed by Complement Fixation (CF) and Agar Gel Diffusion Precipitin (AGDP) Tests

Pig no.		Days postinoculation										Days post-challenge	
		14		20		35		49		64		14	
		AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF	AGDP	CF
260	Original	+	20*	+	20	+	40	+	80	+	80	+	20
	7S	−	10	+	0	+	10	+	80	+	0	+	10
	19S	−	−	−	20	−	10	−	10	−	−	−	−
281	Original	+	40	+	40	+	20	+	20	+	20	+	10
	7S	+	0	+	40	+	20	+	0	+	0	+	10
	19S	+	10	+	40	+	0	−	20	−	0	−	−
406	Original	+	10	+	20	+	10	+	10	+	160	+	160
	7S	+	10	+	20	+	10	+	20	+	160	+	80
	19S	−	0	−	0	−	0	−	0	+	0	−	−

CF — lowest dilution used was 1:10.
AGDP — serum used undiluted.
* — reciprocal of serum dilution.
0 — No fixation at lowest test dilution of 1:10.
− — no visible precipitin lines.
+ — visible precipitin lines.

challenge bleedings. One of the two exceptions to this generalization was pig No 260, which had CF antibody only in the 7S fraction at 14 DPI; in the 20 DPI serum, however, CF activity was only in the 19S zone. Complement fixing antibody was detected in the 19S fraction at the 35 and 49 DPI bleedings but was no longer detectable in the 19S zone in the later and postchallenge bleedings. Conversely, serum from pig No 281 had CF antibody in either or both fractions through 49 DPI. The distribution of AGDP antibody was essentially similar to that observed with the CF activity (Table III).

Antibody was first detected in pig 406 on the 64th day following inoculation with attenuated virus (Table I). This antibody response was coincident with a thermal reaction which peaked at 107.4°F at 61 DPI. The thermal reaction was transitory and no other clinical signs were observed.

DISCUSSION

Assay of antibody response was limited to the results of CF and AGDP tests as classical *in vitro* virus neutralization tests for assay of ASFV antibodies are not available (6). Of the 14 pigs that survived challenge inoculation, 13 had circulating CF antibody and 12 also had AGDP antibodies.

The 14 challenge survivors had no increased antibody following challenge inoculation. As determined by the CF and AGDP tests, 10 of 14 serums had equivalent or lower titers at 14 DPC than at the time of challenge. The serum of pig No 272 had a marked rise in both CF and AGDP antibody after challenge, although this pig was one of four that did not survive challenge inoculation.

The lack of an anamnestic response usually observed following challenge does not appear to be the result of the qualitative aspects of the initial immune response. The primary response in most animals to protein antigens has generally been the formation of a $19S_{\gamma 1}$ antibody that is sensitive to the action of 2-Me. The formation of this antibody subsides in a few days with the appearance of a smaller 7S antibody that is resistant to reductive cleavage by 2-Me.

The data in Tables I through III show that although the antibody response is essentially similar for all pigs, there is considerable qualitative variation in the time of appearance and in the duration of the large 2-Me sensitive 19S antibody and the lack of a secondary response after virus challenge. Reports in the literature are not consistent as to the appearance and duration of the different velocity class antibodies in the primary and secondary antibody response. Tao and Uhr (18), using rat lymph node cell culture, reported that the primary antibody response was pre-

dominantly the formation of 19S antibody, and 7S antibody was the major antibody type in the secondary antibody response. Nossal *et al* (12), however, using rat lymph node cell culture and a different antigen, found a similar 19S antibody in the primary response and the secondary response was 19S antibody. In other instances (5, 9), workers have found both 7S and 19S antibodies present at all times. In the latter example, a response to "booster" injections did not occur. The current concept on the sequential appearance of 19S and 7S antibody formation may need further definition (3, 13) or limitation by host and immunizing agent.

Based on immunogenic competence of the virus, Beveridge (2) has placed ASF in a group of diseases with trachoma, equine infectious anemia, lymphogranuloma, and psittacosis. Hosts infected with one of this group of viruses have an acute attack which, in survivors, is usually followed by a long standing chronic infection. The persistence of ASF infection (7) may provide antigen stimulus for continued antibody production for long periods. The relationship of prolonged course and high titer was reported by Bannister *et al* (1). Under certain conditions of stress, the balance between virus infection and antibody production may be upset, resulting in death or an increased production of antibody. The latter phenomenon of antibody increase was observed in pig No 406, which had a sharp increase in temperature to 107.4F on 61 DPI followed by a significant rise in the serum antibody.

The lack of an anamnestic response to the challenge virus that may be considered as a "booster" dose might be attributed to persistence of virus and intermittent viremia after inoculation with modified virus.

Stone and Hess (17) reported on detection of CF and AGDP antibodies in serums of pigs following inoculation of inactivated ASFV. However, the pigs were generally susceptible to virulent virus challenge. In the present study, pigs that responded with CF and AGDP antibodies following inoculation of attenuated virus were protected against acute ASF following challenge inoculation.

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