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Neutralizing antibodies to African swine fever virus proteins p30, p54, and p72 are not sufficient for antibody-mediated protection

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Abstract

Although antibody-mediated immune mechanisms have been shown to be important in immunity to ASF, it remains unclear what role virus neutralizing antibodies play in the protective response. Virus neutralizing epitopes have been identified on three viral proteins, p30, p54, and p72. To evaluate the role(s) of these proteins in protective immunity, pigs were immunized with baculovirus-expressed p30, p54, p72, and p22 from the pathogenic African swine fever virus (ASFV) isolate Pr4. ASFV specific neutralizing antibodies were detected in test group animals. Following immunization, animals were challenged with 10^4 $TCID_{50}$ of Pr4 virus. In comparison to the control group, test group animals exhibited a 2-day delay to onset of clinical disease and reduced viremia levels at 2 days postinfection (DPI); however, by 4 DPI, there was no significant difference between the two groups and all animals in both groups died between 7 and 10 DPI. These results indicate that neutralizing antibodies to these ASFV proteins are not sufficient for antibody-mediated protection.

Keywords: African swine fever virus; Neutralizing antibodies; Viral proteins

Introduction

African swine fever (ASF) is a highly lethal hemorrhagic disease of domestic swine where mortality rates can approach 100% (Hess, 1982; Maurer et al., 1958). The causative agent, African swine fever virus (ASFV), is currently the sole member of the newly named *Asfarviridae* and is the only known DNA arbovirus (Dixon et al., 2000). In sub-Saharan Africa, ASFV is maintained in a sylvatic cycle among wild swine, warthogs and bush pigs, and argasid ticks of the genus *Ornithodoros*. Unlike domestic swine, ASFV infections of wild swine are asymptomatic with low viremia titers (Plowright et al., 1969). This large natural reservoir of virus poses a constant threat to domestic pig populations worldwide.

There is no vaccine available for ASF. Attempts to vaccinate animals using infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity (Coggins, 1974; Forman et al., 1982; Kihm et al., 1987; Mebus, 1988).

ASF vaccine development is significantly hindered by large gaps in our knowledge of the virus and the complex virus–host interactions involved in infection and immunity. Homologous protective immunity does develop in pigs surviving viral infection. Pigs surviving acute infection with moderately virulent or attenuated variants of ASFV develop long-term resistance to homologous, but rarely to heterologous, virus challenge (Hamdy and Dardiri, 1984; Ruiz-Gonzalvo et al., 1981). Pigs immunized with live attenuated ASF viruses (LAV) containing engineered deletions of specific ASFV virulence/host range genes were protected when challenged with homologous parental virus (Lewis et al., 2000).

Humoral immunity is a significant component of the protective immune response to ASF. ASFV antibodies are sufficient to protect pigs from lethal ASFV infection (Hamdy and Dardiri, 1984; Onisk et al., 1994; Ruiz-Gonzalvo et al., 1981). However, antibody-mediated effector mechanisms associated with the protective response and viral proteins responsible for inducing the response are undefined. Neutralizing antibodies have been described for most viruses and in many cases they have been shown to play a crucial role in a protective response (Dimmock,

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Table 1
Survival, fever response, and viremia of Pr4Δ9GL-immunized pigs following challenge with Pr4[a]

Group	No. surviving	Days to death	Fever			Viremia		
			Days to onset (no. of animals with fever)	Duration	Mean temp.	Days to onset (no. of viremic animals)	Duration	Max titer (log_{10} $TCID_{50}$/ml)
Control (n = 4)	0/4	8.5 ± 0.5	3.5 ± 0.5	6.5 ± 1.5	106.2 ± 0.2	4.0	4.5 ± 0.3	9.1 ± 0.3
Pr4Δ9GL (n = 4)	4/4		15.0 (2)	5.0 ± 0.7	105.7 ± 0.5	10.5 ± 3.5 (2)	14.0	2.9 ± 0.6

[a] Control and Pr4Δ9GL immunized pigs were challenged intramuscularly with 10^4 $TCID_{50}$ of Pr4 at 42 days post immunization.

1984). ASFV neutralizing antibodies directed against three virion proteins p30, p54, and p72 have been described (Borca et al., 1994; Gomez-Puertas et al., 1996; Zsak et al., 1993). And, it has been shown that a neutralizing antibody response to p30 and p54 together provide partial protection to infection (Barderas et al., 2001; Gomez-Puertas et al., 1998).

Here, using immunization and challenge experiments with the African ASFV isolate Pr4, we have examined the role of p30, p54, p72, and p22 in protective immunity.

Results

To evaluate homologous protective immunity to the African ASFV isolate Pr4, pigs were first immunized intramuscularly with 10^4 $TCID_{50}$ of Pr4Δ9GL, an 9GL gene deletion mutant of Pr4 that is attenuated in pigs (Lewis et al., 2000; Zsak et al., unpublished data). Protective immunity was assessed by challenging immunized animals intramuscularly with 10^4 $TCID_{50}$ of the virulent parental strain Pr4 at 42 days post immunization. Solid protective immunity to Pr4 challenge was observed for all Pr4Δ9GL-immunized animals. Here, protection was characterized by 100% survival, the absence of clinical disease, a delayed onset of fever (observed in two of four animals), a delayed onset of viremia (observed in 2 of 4 animals), and a 10^6-fold reduction in maximum viremia titers (Table 1). The level of protection observed here for Pr4 is comparable to that previously reported for the African isolate Malawi Lil20/1 (Lewis et al., 2000) and the pathogenic European isolate E75 (Onisk et al., 1994).

To examine the roles of ASFV proteins p30, p54, p72, and p22 in Pr4 protective immunity, recombinant baculoviruses expressing these proteins were constructed. ASFV p30, p54, p72, and p22 ORFs were amplified from Pr4 genomic DNA template. PCR products were sequentially inserted into a TA cloning vector, PCR2.1, and baculovirus transfer vector, pBlueBac III, and verified by sequence analysis.

Expression of ASFV proteins was evaluated by immunoprecipitation using hyper-immune anti-ASFV swine serum and ^{35}S-methionine pulse-labeled baculovirus-infected Sf21 cell extracts (Fig. 1). Specific ASFV protein bands corresponding to ASFV p72 (lane 2), p30 (lane 3), p54 (lane 4), and p22 (lane 5) were observed. In the control lane (insect cells were infected with a recombinant baculovirus expressing β-galactosidase from the polyhedron promoter), a band corresponding to β-galactosidase was observed. Results were confirmed by Western blot and SDS-PAGE gel analyses (data not shown).

Pigs (n = 6) were immunized with cocktails of the four baculovirus-expressed ASFV proteins as described. Sera from these animals were examined for ASFV antibodies using immunoperoxidase, capture ELISA, and neutralization assays. In peroxidase assays, anti-ASFV antibodies, with titers ranging from 1:80 to 1:1280 were observed (Table 2). Capture ELISA titers ranged from 1:1600 to 1:3200 (Table 2). Virus neutralization titers

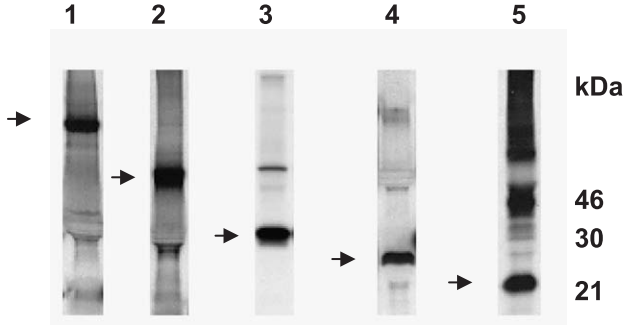

Fig. 1. Expression of ASFV proteins in Sf1 cells infected with recombinant baculoviruses expressing: (lane 1) β-galactosidase-control; (lane 2) p72; (lane 3) p30; (lane 4) p54; and (lane 5) p22. Size markers are shown on the right in kDa.

Table 2
Swine antibody responses following immunization with baculovirus-expressed ASFV structural proteins

Pig no.	Serologic assays[a]		
	Immunoperoxidase	Indirect ELISA	Neutralization
138	1:320	1/3200–1/6400	1/800–1/1600
139	1:1280	1/3200–1/6400	1/800–1/1600
140	1:160–320	1/1600–1/3200	1/800–1/1600
141	1:80	1/1600–1/3200	1/800–1/1600
142	1:320	1/3200–1/6400	1/800–1/1600
143	1:320	1/3200–1/6400	1/1600–1/3200
Hyper-immune	>1:1280[b]	1/3200–1/6400	1/3200–1/6400

[a] These titers represent the range obtained from three independent assays.
[b] End point was not determined.

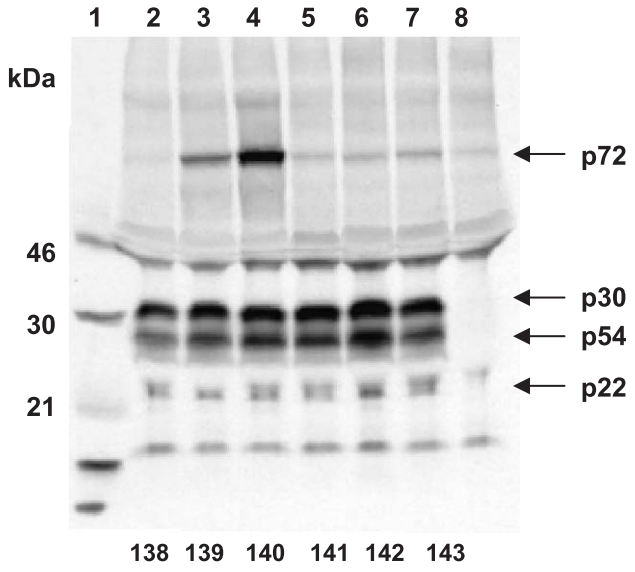

Fig. 2. Western blot analysis of serum from animals immunized with ASFV proteins, p72, p54, p30, and p22. Lanes 2–7 represent serum from immunized animals at 14 days following the last booster immunization. Lane 8, control swine serum. Individual animal numbers are shown below. Size markers (lane 1) are shown in kDa.

ranged between 1:800 and 1:3200. A hyper-immune swine serum (serum from an animal that had survived multiple Pr4 infections) had a neutralization titer of 1:6400 (Table 2). Qualitative assessment of antibody levels against individual ASFV proteins was examined by radio-immunoprecipitation analysis (Fig. 2.). All test sera contained antibodies to p22, p30, p54, and p72. Responses to p22, p30, and p54 were relatively consistent among animals. Variability in response to p72 was observed however it did not correlate with ELISA or neutralizing titer variability.

To assess the role of antibodies to p30, p54, p72, and p22 for protective immunity, immunized pigs were challenged with Pr4 (10^4 $TCID_{50}$). Clinical signs and viremia were monitored. Results from this experiment are shown in Table 3. A significant delay in the onset of clinical disease of approximately 2 days was observed for the immunized group. Onset of viremia was unchanged and apart from a transient decrease in virus titer at 2 DPI for the test group, survival rate and time to death were similar to control group values.

Discussion

Apart from a brief delay in initial disease onset, immunization of swine with p22, p30, p54, and p72 had no effect on disease development, progression, nor outcome (Table 3). Notably, immunized animals had anti-ASFV serologic titers comparable to or higher than those observed for animals successfully protected (Barderas et al., 2001; Gomez-Puertas et al., 1998) or titers present in hyper-immune ASFV swine serum (Table 3). These data indicate that neutralizing antibodies to these viral proteins are not sufficient for mediating protection.

Gomez-Puertas et al. (1998) have reported partial protection for swine immunized with baculovirus-expressed p30 and p54 following challenge with the pathogenic European ASFV isolate E75. Here, 50% of the animals died and clinical disease and significant viremias were evident in most of the survivors. The lack of protection reported here is unlikely to be due to differences in the ASFV challenge model used in the two studies. Notably, in both the E75 and Pr4 challenge models—which differ in virus strains and challenge dose—solid protection characterized by survival, an absence of clinical disease (delayed fever in some cases), and delayed onset and magnitude of viremia is obtained (Onisk et al., 1994). Virulence of the challenge strains used may in part account for the differences. Data are available suggesting that although pathogenic, European ASFV isolates may be more attenuated and adapted for domestic pigs than African field isolates (Mebus, 1988; Ordasalvarez and Marcotegui, 1987). If this is the case, consistent with the results of Gomez-Puertas et al. (1998), partial protection following challenge with a more attenuated virus might be expected.

Together, these data indicate that neutralizing antibodies to these viral proteins are not sufficient to confer protective immunity to viral challenge. And further, they suggest that the relative role of this neutralizing antibody response to antibody-mediated protection may be dependent on the virulence of the ASFV isolate.

Conceivably ASF antibody-mediated protection may be a complex event requiring multiple responses (Ruiz-Gonzalvo et al., 1996) to many different viral proteins, some perhaps involving virus neutralization. In the absence of the whole response, individual contributions of individual protective antigens are difficult to demonstrate experimentally. And, it is possible that additional yet to be discovered

Table 3
Swine fever response and viremia following challenge of p30-, p54-, p72-, and p22-immunized pigs with Pr4

Group	Number surviving	Days to death	Fever	Viremia			
			Days to onset	Days to onset	Mean titer $\log_{10}$ $TCID_{50}$/ml		
					2 DPI	4 DPI	7 DPI
Test	0/6	9.2 (0.5)*	4.0 (0.0)	2.0 (0.0)	5.6 (0.1)	8.3 (0.3)	8.8 (0.2)
Control	0/4	9.0 (0.0)	2.3 (0.3)	2.0 (0.0)	7.4 (0.3)	8.7 (0.1)	9.3 (0.3)

* Means (with standard errors).

neutralizing epitopes play critical roles in protection. However, it is much more likely that other effector mechanisms apart from virus neutralization are important for antibody-mediated protection.

Other in vitro cytolytic effector functions mediated by anti-ASFV antibody have been described; however, no significant correlation between complement-dependent antibody lysis and antibody-dependent cell-mediated cytotoxicity antibody titers in vitro and protective immunity have been demonstrated (Norley and Wardley, 1982; Norley and Wardley, 1983). Interestingly, anti-ASFV antibodies have been shown to have novel inhibitory effects on ASFV replication (DeTray, 1957; Malmquist, 1963; Mebus, 1988; Onisk et al., 1994; Schlafer et al., 1984a, 1984b). This phenomenon was first described in macrophage cell cultures by Malmquist (1963) and later by Coggins et al. (1968). The continuous presence of convalescent serum (at nearly undiluted concentrations) protected autologous buffy-coat cell cultures from infection with homologous but not heterologous ASFV strains. This monocyte infection-inhibition (M-II) activity was mediated by the purified IgG fraction and was effective in inhibiting viral replication after virus adsorption had occurred (Ruiz-Gonzalvo et al., 1986a, 1986b). And notably, M-II antibody titers correlated with protection from challenge (Knudsen et al., 1987; Ruiz-Gonzalvo et al., 1986b). We are currently assessing the role of M-II antibodies in protective immunity and identifying viral proteins that induce them.

Materials and methods

Virus strains and cell culture

The pathogenic ASFV isolate Pr4 (Kleiboeker et al., 1998), the attenuated recombinant Pr4 9GL gene deletion mutant (Pr4Δ9GL) (Zsak et al., unpublished data), and Vero cell culture adapted virus BA71V (kindly provided by Jose M. Escribano INIA, Madrid, Spain) were used in these experiments. Insect cell lines SF21 and High Five (Invitrogen, Carlsbad, CA) were propagated in Grace's Insect Media (Invitrogen) and Ex-cell 400 Media (JRH Biosciences, Inc., Lenexa, KS) supplemented with 5% fetal bovine serum (FBS) and incubated at 27 °C. Vero cells were obtained from American Type Culture Collection (ATCC), propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C under 5% CO_2. Primary porcine macrophage cell cultures were prepared from heparinized swine blood as previously described (Neilan et al., 1997).

PCR and DNA sequencing analysis

Genes encoding p30, p54, p72, and p22 were amplified, by PCR, using genomic DNA prepared from PR4. Specific primers selected for each gene were modified to create unique restriction sites at the 5′ and 3′ ends, respectively.

Primer pairs were:

p30
Forward: 5′-AGAGGTTGAAGAT**CCATGG**TTACCCATT-3′ (*Nco*I)
Reverse: 5′-CTAATAAATCT**GGATCC**TGCTGCTGCAG-3′ (*Bam*HI)
p54
Forward: 5′-CTTATAATATA**CTGCAG**TATGTTGAGTC-3′ (*Pst*I)
Reverse: 5′-TTCTTGA**GGATCC**TTGGAAAGTTGGTCC-3′ (*Bam*HI)
p72
Forward: 5′-TATCA**GGATCC**TTCGCATAAACCGCCA-3′ (*Bam*HI)
Reverse: 5′-GGAAGCCCAC**AGATCT**AACCCATTGTG-3′ (*Bgl*II)
p22
Forward: 5′-CAGAAA**GGATCC**AATATTATGTAGACC-3′ (*Bam*HI)
Reverse: 5′-CGATGCACAATATTAT**AAGCTT**TAAACCG-3′ (*Hin*dIII)

PCR was performed for 40 cycles of thermal denaturation (96 °C for 15 s), re-annealing (50 °C for 30 s), and extension (60 °C for 30 s) with AmpliTaq DNA polymerase (Perkin-Elmer, Roche, NJ). Amplified products were cloned into the TA cloning vector, pCR2.1 (Invitrogen), and cloned inserts were verified by sequence analysis using the chain termination method (Sanger et al., 1977).

Construction of recombinant baculoviruses

Cloned PCR products were digested with appropriate enzymes and inserted in the multiple cloning site of the baculovirus transfer vector, pBlueBac III (Invitrogen). Recombinant pBlueBac III plasmid DNAs were purified and sequenced to ensure sequence fidelity and correct orientation for expression. Co-transfection experiments were performed using a commercial transfection kit according to the manufacturer's protocol (Invitrogen). Recombinant, β-galactosidase-expressing plaques were selected and plaqued purified three times to homogeneity.

Immunoprecipitation

For detection of ASFV gene expression, recombinant baculovirus and mock infected insect cells (Sf21) were labeled with ^{35}S-methionine. Infected cells were lysed in lysis buffer and incubated with hyper-immune anti-ASFV swine sera for 1 h. Immune complexes were precipitated by the addition of protein A-coated Sepharose CL-4B (Sigma, St. Louis, MO). Immunoprecipitates were washed four times in lysis buffer, solubilized by boiling in loading buffer, and analyzed by 10–20% gradient SDS gel electrophoresis (Novex, San Diego, CA) using autoradiography.

For detection of antibodies from immunized pigs, ASFV-infected and mock-infected swine macrophage or Vero cell cultures were pulse-labeled with ^{35}S-methionine, lysed, and incubated with test pig sera as described above.

Capture ELISA

Monospecific antisera from rabbits immunized with baculovirus-expressed p22, p30, p54, or p72 were combined and used as capture antibody. The pooled antisera were diluted 1:400 in a 0.05 M carbonate buffer (pH 9.6) and allowed to coat Immulon 2 plates overnight at 4 °C. ASFV antigen was prepared by inoculating roller bottles containing confluent Vero cells with Ba71V (MOI = 1) and harvesting when cultures reached 90–100% CPE. Antigen was semi-purified by centrifugation on 30% and 60% sucrose step gradients at 15,000 × *g*. Antigen was diluted 1:100 in blocking buffer (Milk diluent, KPL, Inc, Gaithersburg, MD) and incubated for 1 h at 37 °C in a humidified chamber. Plates were washed twice with wash buffer (KPL). Duplicates of swine sera were diluted 1:100–1:4800 in blocking buffer and incubated for 1 h at 37 °C. Plates were washed five times with buffer and incubated with peroxidase-labeled anti-swine antiserum (KPL) for 1 h at 37 °C. Plates were washed six times with wash buffer and incubated with ABTS substrate and peroxide as directed by the manufacturer. The reaction was stopped with 1% SDS and the optical density was read at 405 nm. End-point titers for test sera were determined as the dilution of serum giving an optical density reading that was 1.5× greater than the control sera pool (obtained from animals immunized with wild-type baculovirus only).

Neutralization test

Serum neutralizing titers were determined using an infectious focus assay (Zsak et al., 1993). Briefly, heat-inactivated swine sera (1 h at 56 °C) were diluted (1:5–1:6400) in RPMI media with 10% heat-inactivated fetal bovine serum and 0.05% Tween-80. Pr4 stock virus (10^8 $TCID_{50}$/ml) was sonicated, spun at 3000 rpm, adjusted to 0.05% Tween-80, and passed through a 0.45-μm filter (Millipore). Clarified virus, approximately 100 PFU diluted in RPMI–0.05% Tween-80, combined with swine sera (at varying 2-fold dilutions), was incubated overnight at 37 °C. The virus was added to macrophages and incubated overnight at 37 °C. Infected cells were fixed with ice-cold methanol and processed for immunoperoxidase staining using 135D4 monoclonal antibody which detects ASFV protein p72 (Zsak et al., 1993). End-point titers of ASFV test sera are expressed as dilutions of sera giving greater than 50% fewer infected cells compared to control sera obtained from animals immunized with baculovirus only.

Swine immunization and infection

Four pigs were immunized intramuscularly with 10^4 $TCID_{50}$ of Pr4Δ9GL, a Pr4 9GL gene deletion mutant attenuated in pigs (Lewis et al., 2000; Zsak et al., unpublished data). Immunized animals were challenged intramuscularly with 10^4 $TCID_{50}$ of parental Pr4 at 42 days post immunization. Clinical signs of ASF (rectal temperature greater than or equal to 40 °C, anorexia, lethargy, shivering, cyanosis, and recumbency) were monitored daily. Blood samples were collected every other day postinfection (DPI). Virus titration of blood samples was performed as previously described (Onisk et al., 1994). Virus titers were calculated using the method of Spearman–Karber and expressed as $TCID_{50}$ (Finney, 1984).

Ten pigs (20–30 kg) were divided into two groups for protein immunization experiments: the test group ($n = 6$) was immunized intramuscularly with a cocktail of recombinant baculovirus-infected insect cell extracts containing p30, p54, p72, and p22, while the control group ($n = 4$) was immunized with the parental baculovirus-infected insect cell extracts. Each animal dose contained $1–2 \times 10^8$ Sf21 cells or about 200 μg total protein emulsified in Freund's complete adjuvant for the primary inoculation and incomplete adjuvant for additional boosters administered at 4-week intervals. Fourteen days following the fourth booster immunization, pigs were challenged intramuscularly with 10^4 $TCID_{50}$ of ASFV Pr4 and monitored as described above.

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