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Characterization of a Soluble Hemagglutinin Induced in African Swine Fever Virus-Infected Cells

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Hemadsorption (Had) of erythrocytes to the surface of African swine fever virus (ASFV)-infected cells is a well-known phenomenon but hemagglutination of pig erythrocytes in the supernatant of ASFV-infected cells has not been reported before. We report here the discovery of a pig erythrocyte-agglutinating activity released to the *in vitro* cell culture medium by cells infected with some isolates of ASFV. This finding allowed the identification and characterization of a soluble hemagglutinin (HA) molecule that could be separated from the ASFV particles either by ultracentrifugation or by gel-permeation chromatography. The HA was inactivated by agents known to affect protein conformation such as heat, β-mercaptoethanol, urea, and guanidine isothiocyanate. Glycosilation seemed to be of importance since treatment of HA with glycosidase F inhibited the hemagglutinating activity and HA could be partially purified by affinity chromatography on immobilized concanavalin A. When native it had an estimated molecular weight of 300 kDa by gel-permeation chromatography yielding 51-kDa protein monomers under denaturing conditions as identified by immunoblotting. Preliminary attempts to correlate the induced anti-HA serum antibodies with viremia or infection–inhibition serum antibodies after infection of pigs with attenuated ASFV or immunization with purified HA are also reported.

INTRODUCTION

African swine fever virus (ASFV) is an icosahedral cytoplasmic deoxyvirus (family Iridoviridae) similar to poxvirus which infects porcine species. The lack of a vaccine against ASFV and its high mortality rate make this infection the most damaging to the international swine industry (Wardley and Wilkinson, 1985). Attempts to define immunizing agents that would induce protection against ASFV challenges in the absence of viremia (to avoid the generation of carrier animals) have failed (Kihm *et al.*, 1987). A characteristic of the ASFV is the induction of hemadsorption (Had) or binding of pig erythrocytes to the cell surface of virus-infected pig macrophages, a property widely used for diagnostic purposes (Malmquist and Hay, 1960). However, despite numerous studies on the molecular biology of the ASFV, neither the identification of the virus-coded molecule causing Had nor the possible hemagglutination (Hag) of free erythrocytes induced by ASFV infection has yet been described.

In this report we describe a virus-induced glycoprotein (HA) that is released soluble to the cell culture medium by ASFV-infected cells and produces *in vitro* hemagglutination of pig erythrocytes in the absence of ASFV particles. Pigs surviving infection with attenuated ASFV or immunized with partially purified HA developed Had-inhibition, Hag-inhibition, and infection–inhibition serum antibodies. The possible significance of these data to the development of an ASFV vaccine is also discussed.

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MATERIALS AND METHODS

Cell cultures and African swine fever virus isolates

To prepare pig buffy coat cultures, cells from defibrinated blood in donor serum were incubated at 37° during 2–3 days and inoculated with the ASFV. Vero (VR, ATCC-CCL81) and monkey stable (MS) cells were grown at 37° in culture medium made of Eagle's MEM containing 10% bovine fetal serum, 50 μg/ml gentamicin, and 50 IU/ml micostatin. The ASFV isolates (Table 1) were isolated in pig buffy coat cultures and detected by Had (Malmquist, 1960a,b) or direct immunofluorescence (E3158) (Gonzalvo *et al.*, 1986a,b). The isolates were then propagated in VR or in MS cells throughout a number of passages (Table 1, number after VR or MS).

Hemagglutination assay

The Hag assay was carried out in U-bottom microtiter plates by diluting the sample in serial two-fold dilutions in 50 μl of HA buffer (0.1 *M* sodium acetate, 1 m*M* $MnCl_2$, 1 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 1 *M* NaCl, pH 6.8) supplemented with 0.1% of fetal calf serum and then mixing each dilution with 25 μl of pig erythrocytes (obtained in Alsever's solution and stored at 4°) at 0.2% in

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HA buffer. The mixtures were agitated and then incubated at 37° during 4 hr for optimal reading. One HA unit was defined as the reciprocal of the highest dilution showing Hag.

Hemagglutinin preparation

Confluent cultures of VR cells in flasks of 150 cm^2 or roller bottles (Linbro Dua-Cult, Flow Labs, Ayrshire, Scotland) were inoculated with 0.5–1 ASFV (E1207-VR11) per cell and then absorbed during 2 hr in 3–5 ml, the inoculum were removed, cultures were washed three times with Dulbecco's phosphate-buffered saline (PBS), and 50 ml/flask or 200 ml/bottle of cell culture medium with 1% fetal calf serum was added. The culture was harvested after 3–4 days at 37° and centrifuged at 12,000 *g* for 30 min resulting in a supernatant and a pellet. The supernatant was dialyzed against 20 m*M* $(NH_4)HCO_3$, lyophilized, and resuspended in 1/100 of the original volume of HA buffer (cell-free HA). The pellet was washed three times with PBS, resuspended in 1/100 of the original volume of HA buffer, and sonicated until cell lysis was complete, and the supernatant after centrifugation was harvested (cell-associated HA).

The cell-free HA and the cell-associated HA were separately ultracentrifuged through a cushion of 20% (w/v) sucrose in HA buffer at 100,000 *g* for 2 hr to eliminate the ASFV particles (ASFV in the ultracentrifuged pellet was 10^{10} $TCID_{50}$ per 10^6 U of HA). Any residual viral infectivity (0.1% of the initial titer) in the supernatants (HA concentrates) was eliminated with 0.05% β-propionolactone during 2 hr. Noninfected VR cell cultures (Hag-negative controls) were processed as above.

To estimate the Hag activity of the ASFV particles, the ultracentrifuged pellets and the sucrose cushion were mixed, sonicated, 10-fold diluted with HA-buffer, and then ultracentrifuged. The supernatants from the first and second ultracentrifugations and the final pellet were independently assayed for Hag activity. Results were expressed as a percentage of recovered Hag activity in the pellet by the formula Hag activity in the pellet/Hag activity in the pellet + Hag activity in the supernatants × 100. Recovery of the initial Hag activity before ultracentrifugation (Hag activity of pellet + supernatants/initial Hag activity × 100) was 100%.

Twenty-five milliliters of HA concentrate (Table 3) were chromatographed through a Sepharose–concanavalin A (Con A) (Pharmacia, Sweden) column (1.5 × 1 cm) previously washed with 200 ml of HA buffer. The HA concentrate was passed 8 times through the column with the aid of a peristaltic pump at room temperature. After washing the column with 200 ml of HA buffer, 50% of the bound Hag was eluted with 10 ml of 1 *M* methyl-α-D-glucopyranoside and 1 *M* methyl-α-D-mannopyranoside in HA buffer by passing the solution 10 times through the column with the aid of a peristaltic pump.

Physicochemical treatments of HA

The supernatant from E1207-VR11-infected VR cell cultures was concentrated 100-fold by dialysis and lyophylization and most of the ASFV was eliminated by ultracentrifugation as described above. All the physicochemical treatments were performed with 2000 U of soluble HA during 30 min (except glycosidase F) with the final concentrations shown in Table 2. After treatment, the HA was dialyzed against PBS.

The Hag activity after 60 hr of incubation with Glycosidase F (Boehringer-Mannheim, Germany) (a 30-min incubation had no effects) was expressed relative to the Hag activity of HA incubated at 37° or at 4° (same results) during the same length of time but in the absence of the enzyme.

Concanavalin A was added to HA at different concentrations, mixtures were centrifuged after 30 min, pellets were discarded, and supernatants were assayed. Table 2 shows the optimal results obtained at 3 mg/ml.

To estimate the possible oxidation of the polypeptide backbone during the metaperiodate treatment, horseradish peroxidase (RZ = 3,3; Boehringer-Mannheim) (EC 1.11.1.7), an enzyme preparation having a low carbohydrate content (Tijssen, 1985), was held in 0.04 *M* sodium metaperiodate for 30 min. After dialysis and enzyme assay, 87.5% of the initial peroxidase activity could be recovered (the activity of the peroxidase was measured as described in Coll, 1987).

Hag residual activity after treatment was expressed as a percentage of the initial activity by the formula Hag activity recovered after treatment/Hag activity before treatment × 100.

Hemagglutination-inhibition assay

To estimate anti-HA antibodies titers in pig serum, the pig serum was inactivated to destroy the complement by heating at 56° for 30 min and adsorbed with pig erythrocytes at 37° for 1 hr. The erythrocytes were removed by centrifugation and the supernatant was mixed with 4 vol of kaolin. After incubation for 60 min at room temperature and centrifugation, the supernatant was removed and used for the assay. Eight U of HA in a 25-μl volume of HA buffer and 25 μl of serial two-fold dilutions of the inactivated–adsorbed serum in HA buffer were mixed, incubated, and read as in the Hag assay.

Infection–inhibition assay

To estimate the titers of ASFV neutralizing antibodies in pig serum, the culture medium of the pig buffy

coat culture was aspirated after 3 days of incubation and 200 μl of ASFV diluted in immune pig or in donor pig serum was added per well. Dilutions of the virus were in a 10-fold series and four parallel wells were used per dilution. The microtiter plates were then incubated at 37° for 7 days and examined once a day for the cytopathic effect. Results of the titrations were expressed as tissue culture infectious dosages at 50% ($TCID_{50}$). The infection–inhibition index was defined as the difference between the reciprocal of the log of the control serum titration and the reciprocal of the log of the immune serum titration (Gonzalvo *et al.*, 1986a,b).

Immunofluorescence

To estimate total anti-ASFV titers in pig serum, ASFV-infected VR cells on coverslips were washed three times in PBS and fixed in acetone for 15 min. Then they were incubated for 1 hr with several dilutions of the immune pig sera, washed, and incubated with a protein A–FITC conjugate (Gonzalvo *et al.*, 1986a,b).

Enzyme immunoassay (ELISA)

To estimate anti-HA pig antibodies by ELISA, microtiter plates (Dynatech, Plochingen, W. Germany) were coated to dryness with 1000 U of purified HA, washed, and kept dried for weeks (Martinez and Coll, 1988). Plates without any coating were used for controls. Horseradish peroxidase-conjugated protein A (Nordic, Tilburg, The Netherlands) was used to develop the reaction between the pig anti-HA serum and the HA-coated solid phase.

Immunization of pigs by injection with attenuated ASFV and challenge with virulent ASFV

Pigs were intramuscularly injected three times, once every month, with the cytoplasmic fraction of 3×10^8 MS cells infected with E70-MS14 (first in complete Freund's and then in incomplete Freund's). One month after the last injection, the pigs were bled and then challenged by introducing into the same room a pig infected 3 days before with virulent E70 virus. Viremia was estimated in blood samples taken every 7 days by pig buffy coat cultures as described (Gonzalvo *et al.*, 1986a,b).

Immunoblotting of serum from ASFV-infected/survivor pigs and HA-immunized/challenged pigs

Serum was obtained from a control nonimmunized pig, a pig surviving infection with the attenuated ASFV isolate E1207-VR11 (Gonzalvo *et al.*, 1986a,b), and a pig surviving immunization with HA and the challenge with the virulent E1207.

The concentrated HA obtained from VR cells infected with E1207-VR11 was treated with 0.05% β-propionolactone (lack of infectivity was confirmed by inoculation of 1/10 of the total HA in VR cell culture and observation during 15 days). Two pigs were immunized with HA bound to Sepharose–Con A beads (5×10^6 units of HA per pig per injection, two injections per pig, once per month) by both intramuscular and intraperitoneal injections (hypothetically, the Con A–Sepharose beads would work as adjuvants). One month after the second injection, the HA-immunized pigs were placed in the same room with one pig infected by intramuscular injection of 10^7 $TCID_{50}$ of virulent E1207. The E1207-infected pig died 5 days after the injection, one of the HA-immunized pigs died after 12 days, and one of the HA-immunized pigs survived. Blood viremia by sampling every 2 days could not be demonstrated in the surviving pig but it had a fever from the 11th to the 17th day after the challenge. The surviving pig was bled 1 month later for serum.

Detection of anti-ASFV antibodies was made by immunoblotting as described (Pastor *et al.*, 1989). Briefly, soluble ASFV cytoplasmic proteins were obtained in MS cells infected with E70-MS81 at a multiplicity of infection of 10 and resolved in 17% polyacrylamide gel electrophoresis (PAGE) after denaturation by β-mercaptoethanol and sodium dodecyl sulfate. After transfer to nitrocellulose filters, they were dried, cut into 0.5-cm-wide strips (containing 10 μg of ASFV each), and allowed to react with 30-fold diluted pig sera in 2% nonfat dry milk in PBS. After washing, the immunocomplexes were detected by reaction with protein A–peroxidase using 4-chloronaphthol as substrate (Alcaraz *et al.*, 1989).

RESULTS

Detection of Hag in the cell culture medium from ASFV-infected cells

During the screening of ASFV-infected pig buffy coat cultures by Had, the E1207 isolate, which also produced visible Hag of the free pig erythrocytes present in the supernatant of the cultures, was found (never seen before). Since passages of ASFV in cell line cultures also produced Had and cytopathic effects (Malmquist, 1962, 1963), the E1207 isolate was adapted to VR cells. After 11 passages, the Hag titer of E1207-VR11 Vero-infected supernatants (cell-free or soluble HA) varied between 4 and 8 HA units/ml. Further work on the buffer for the Hag reaction resulted in an optimal buffer (HA buffer) of high ionic strength with which titers up to 300 HA units/ml could be obtained (Table 1) After infection with a multiplicity of infection of 1 $TCID_{50}$ per VR cell, the soluble Hag titers increased from 12 to 25 to 100% of the highest Hag titer at 1, 2, and 3 days after infection, respectively. Four days after infection the VR cells were rounded and floated but no further increase in Hag titer could be detected in the

TABLE 1

IN VITRO PRODUCTION OF HA BY VR CELLS INFECTED WITH ASFV ISOLATES FROM SPAIN DETECTED BY HAD OR IMMUNOFLUORESCENCE

Isolates	Cell culture	Had	Total HA Units produced	
			Supernatant	Pellet
E3158	VR7	−	0	0
E1136	VR11	+	16000	51200
E608	VR11	+	0	12800
E3394	VR14	+	0	51200
E1207	VR11	+	8000	51200
E70	MS14VR6	+	16000	51200
E608	VR83	+	0	25600

Note. ASFV cell line adapted isolates E3158-VR7, E1136-VR11, E608-VR11, and E3394-VR14 caused death of all pigs inoculated with them, 7 to 15 days after infection. ASFV isolate E1207-VR11 caused death in 50% of the pigs inoculated with it. ASFV isolates E70-MS14VR6 and E608-VR83 did not kill any of the pigs inoculated with them nor produced viremia. *In vitro* production of HA was estimated 3 days after ASFV infection (multiplicity of infection = 0.1) of VR cell monolayers in 150-cm^2 flasks with 50 ml of DMEM supplemented with 10% fetal calf serum. After the cytopathic effect, the cell suspensions were centrifuged at 12,000 *g* for 30 min, and the supernatants (cell-free or soluble HA) were dialyzed against 50 m*M* ammonium bicarbonate, concentrated by lyophylization, and resuspended in 5 ml of HA-buffer before the HA estimation. The cellular pellets (cell-associated HA) were also resuspended in 5 ml of HA buffer, sonicated, and centrifuged to eliminate cell debris before the HA estimation.

supernatant. The cellular pellet obtained from the infected VR cell cultures after being centrifuged and sonicated also produced Hag (cell-associated HA) (Table 1).

Production of HA by different ASFV isolates

To study the production of HA by different ASFV isolates, seven isolates were selected differing in year, geographical area of isolation, production of Had, *in vivo* virulence, and number of passages in cell culture (Table 1). The E3158-VR7 isolate was unique in that it was both Had- and Hag-negative, while all the other studied isolates were capable of producing cell-associated Hag. Soluble Hag could be detected in the supernatants from VR cells infected with the isolates E1136-VR11, E1207-VR11, and E70-MS14VR6. The isolate E608 produced about the same amount of cell-associated HA whether in the 11th (12,800 U of HA) or in the 83rd (25,000 U of HA) passages in VR cell culture. Furthermore, no correlation could be obtained between the cell-associated (pellet) or the cell-free (supernatant) production of HA and either the virulence (percentage of pigs killed after *in vivo* infection) or the viremia (virus titer in peripheral blood after *in vivo* infection) induced by the ASFV isolates studied (legend to Table 1). All the Hag activity obtained from the different isolates could be inhibited by pig serum obtained from pigs infected with E1207-VR11 (not shown). The ASFV-infected (1 $TCID_{50}$ per cell) VR cell protein patterns were identical for all the isolates mentioned above and similar to the ones described before (Tabares *et al.*, 1980a,b; Escribano and Tabares, 1987; Alcaráz *et al.*, 1992) as demonstrated by 5-day labeled infected VR cell cultures with ^{14}C-labeled amino acids (Amersham, 10 μCi per well per 100 μl) (not shown).

Partial purification and characterization of the soluble HA

To further study the properties of the virus-induced soluble molecule producing Hag (HA), supernatants from the E1207-VR11 VR-infected cell cultures were obtained in larger amounts, concentrated 100-fold by lyophylization, and ASFV particles were removed by ultracentrifugation. No significant loss of Hag activity was detected after any of these procedures. The lyophylized concentrated HA was stable when kept dry for 1 year at 4°. A series of physicochemical treatments were performed on the redisolved concentrated HA preparation and the agent was removed by dialysis before the estimation of the residual Hag activity. Table 2 shows that the Hag activity was sensitive to agents known to affect the conformational structure of proteins such as heat, β-mercaptoethanol, urea, and guanidine isothiocyanate. On the other hand, the Hag activity was also sensitive to agents modifying carbohydrates such as glycosidase F and sodium metaperiodate. Although the metaperiodate could also affect the protein, this effect appears to be minimal under the conditions used (see Materials and Methods). In addition, the Hag activity could be removed by precipitation with the lectin concanavalin A. Taken together, all these results suggest that the Hag activity resides in a

TABLE 2

EFFECT OF PHYSICOCHEMICAL TREATMENTS ON SOLUBLE HAG ACTIVITY

Treatment	Concentration	Hag activity (%)
Ultracentrifugation	—	95
100° 2′	—	0
β-mercaptoethanol	0.1M	7
Urea	2M	35
Guanidine isothiocyanate	0.5M	25
Glycosidase F, 37°	100 Units/ml (60 hr)	25
Sodium metaperiodate	0.04 *M*	0
Concanavalin A supernatant	3 mg/ml	0
Methyl-α-D-mannopyranoside	1 *M*	100
Methyl-α-D-glucopyranoside	1 *M*	100
Ethylenediamine, pH 4	0.05 *M*	100
Sodium acetate, pH 4.5	0.1 *M*	100
β-propionolactone	0.05%	100

Note. Hag activity was expressed as the percentage of the initial activity derived from the formula, Hag activity recovered after treatment/Hag activity before treatment × 100.

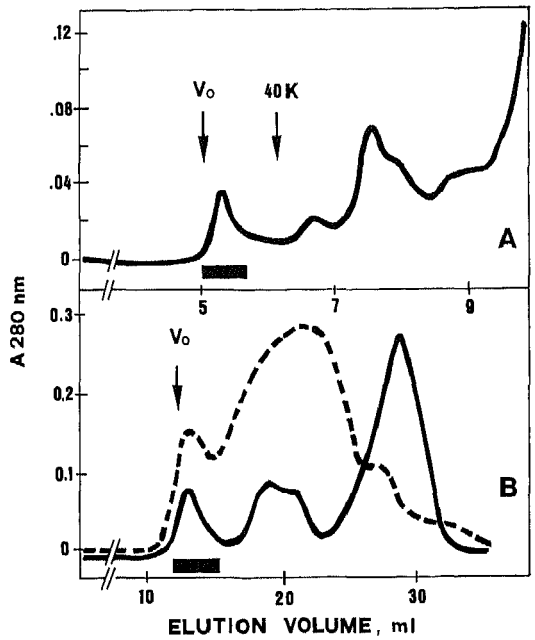

FIG. 1. Supernatant from ASFV (E1207-VR11)-infected VR cell cultures separated by gel-permeation chromatography by HPLC (A) or by Sephacryl S-300 (B). Supernatants were dialyzed, lyophylized, and redisolved 100-fold concentrated. The chromatography by HPLC was performed in an HPLC-Waters apparatus equipped with a protein-pak 300SW column at 0.5 ml/min in PBS. The amount injected was 250 μl. The chromatography over Sephacryl S-300 was performed in a 50 × 1 cm column in 1 *M* ammonium bicarbonate. The amount separated was 1.5 ml. The molecular weight markers used were V_0, blue dextran (2000 kDa); P, peroxidase (40 kDa). Horizontal black bars represented the Hag positive fractions. (-----) HA extracts obtained in 10% fetal calf serum containing medium. (——) HA extracts obtained in 1% fetal calf serum containing medium.

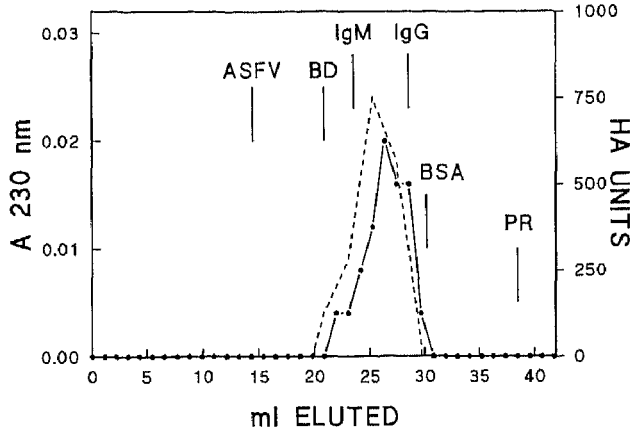

FIG. 2. Gel-permeation chromatography over Sepharose 4B of partially purified soluble HA. The fractions obtained from Sephacryl S-300 containing Hag activity (Fig. 1B) were pooled and chromatographed in a 50 × 1 cm column in 1 *M* ammoniumn bicarbonate. Molecular weight markers used were BD, blue dextran (2000 kDa); IgM, trout immunoglobulin (750 kDa); IgG, mouse immunoglobulin G (150 kDa); BSA, bovine serum albumin (68 kDa); PR, phenol red; ASFV, african swine fever virus purified by ultracentrifugation and heat-inactivated by 2 min at 100°. The V_0 theoretical exclusion volume was 14 ml. (●) HA units; (-----) OD at 230 nm.

glycoprotein(s) and that glycosilation is important for its activity. The native concentrated HA was excluded from Sephacryl S-300 and protein-pak 300SW (high-pressure liquid chromatography or HPLC) (Fig. 1) but retained in Sepharose 4B (Pharmacia, Uppsala, Sweden) (Fig. 2) with an apparent molecular weight of 300 ± 50 kDa. The gel chromatographies had to be performed in 1 *M* ammonium bicarbonate buffer, pH 8.8, since the HA stuck to the columns at a lower ionic strength.

After reultracentrifuging the cell-associated HA (1,500,000 U), 6.9% of the recovered Hag activity was still associated with the ASFV-containing pellet. Since this could be due to contamination with cellular host membrane vesicles (Carrascosa *et al.*, 1985), the possibility of HA completely separate from ASFV was studied by analyzing the ultracentrifuged pellet from soluble HA (900,000 U). However, in this case, 4.7% of the initial Hag activity could be detected in the ASFV-containing pellet (Table 2).

Table 3 shows a representative purification chart of the soluble HA from 3-day supernatants of VR cells infected with E1207-VR11 by concentration (dialysis, lyophilization, resuspension, and elimination of the precipitate) followed by affinity chromatography over a column of Con A immobilized on Sepharose beads. Total recovery of the initial Hag activity was mostly dependent on the percentage of elution of Hag in the affinity column, since the elution of the HA bound to the Con A–Sepharose column (3,000,000 U of HA bound to 3 ml of Con A–Sepharose) was obtained with difficulty. No more than 10% of the initial input Hag activity could be eluted by each of 1 *M* manopyranoside, Ca^{2+}- and Mg^{2+}-free PBS, 1 *M* NaCl, 50 m*M* ethylenediamine, pH 11, HA buffer containing 30 m*M* EDTA, 1% Tween 20, 1% SDS, 1% octylglucoside, 1% Triton X-100, 2 *M* urea, or pH 4.5. However, 0.8 *M* glucopyranoside was capable of eluting about 25–50% of the initial bound Hag activity, depending on the experiment (Fig. 3). Maximal elution of 84% of the initial Hag activity could be obtained by recycling 60 ml of 1 *M* glucopyranoside, 1 *M* manopiranose in HA buffer overnight (≥8 cycles). Maximal concentrations obtained in the elu-

TABLE 3

PURIFICATION OF SOLUBLE HA FROM SUPERNATANTS OF VR CELL CULTURES INFECTED WITH E1207-VR11

Fraction	Volume (ml)	Hag activity per ml × 10^{-5}	Hag activity Recovery (%)
Supernatant	1250.0	0.003	100
Concentrate	25.0	0.12	80
Concanavalin A	0.5	3.9	52

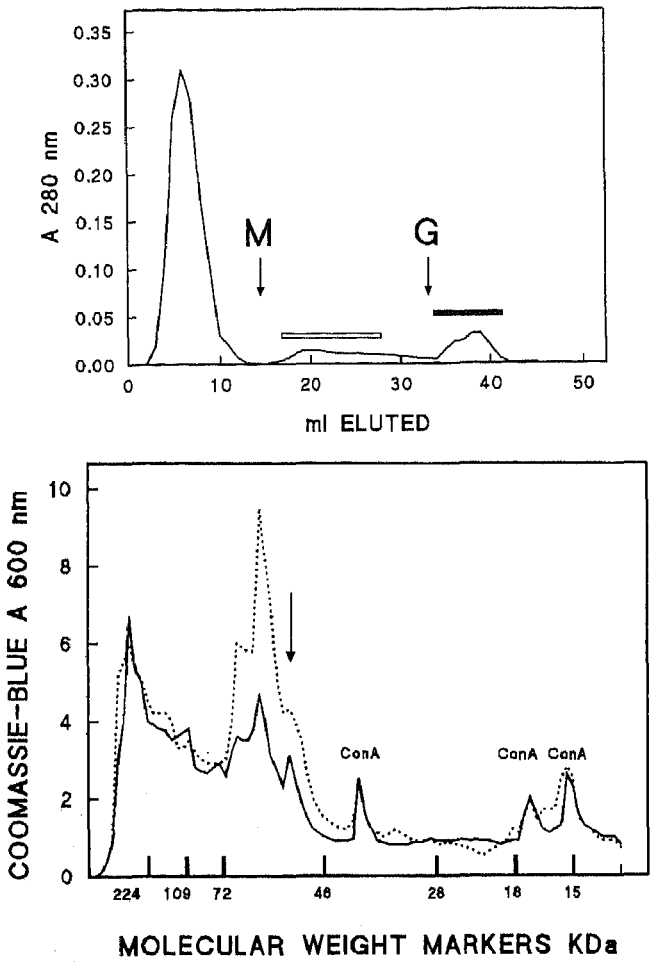

FIG. 3. Affinity chromatography over Con A–Sepharose of concentrated supernatant (upper) and Coomassie blue profile of the bands separated by PAGE (lower). Ten milliliters of concentrated HA was chromatographed over a column (1.5 × 1 cm) of Sepharose–Con A and 1-ml fractions were collected. After washing the HA was eluted with 1 *M* methyl-α-D-mannopyranoside (fraction 19 to 29 *M* fractions) or 1 *M* methyl-α-D-glucopyranoside (fractions 30 to 42 G fractions). Recovery of Hag activity ranged from 25 to 60% in the G fractions (horizontal black bar). The fractions M and G were separately pooled, dialyzed, lyophilized, resuspended in 200 μl of 20 m*M* ammonium bicarbonate, and 20 μl separated by PAGE on a 6 to 20% polyacrylamide gradient gel. After staining with Coomassie blue, the bands were scanned at 600 nm. The position of molecular weight markers is given in the horyzontal axis. The scans were aligned by the position of the Con A bands (labeled in the lower figure as Con A) identified with the help of paralleled-run purified Con A. (——) PAGE profile of G fractions (Hag-positive fractions). (-----) PAGE profile of M fractions (Hag-negative fractions). The vertical arrow indicates the position of the 51-kDa protein.

tion buffer were 2000–5000 U HA/ml. When this purified HA was dialyzed to remove salts and sugars, only ≤1% of the starting Hag activity could be further recovered.

A 51-kDa protein band increased in the Hag-containing fractions obtained by Con A–Sepharose chromatography by PAGE (arrow, Fig. 3, bottom graph). PAGE of the Con A–Sepharose beads containing bound HA showed intense bands at the 50- to 55-kDa region and faint bands at about 180, 220, and 290 kDa with their relative proportions variable from sample to sample. By including 5 *M* guanidinium isothyocianate in the PAGE sample buffer, the high-molecular-weight bands (180, 220, and 290 kDa) dissappeared and only a wide and distorted band at the 50- to 55-kDa region remained which did not appear in control noninfected supernatants treated in the same way (not shown). Three more bands (at 40, 17, and 15 kDa) were always present due to Con A leaking from the column as shown by parallel PAGE of purified Con A (Fig. 3) or by lack of radioactivity in the above-mentioned three bands when the HA was purified from ASFV-infected cells incubated in the presence of ^{14}C-labeled amino acids.

Anti-HA antibodies in ASFV-immunized and ASFV-infected pigs

To evaluate the possible biological significance of the HA and/or of the antibodies against HA, several *in vivo* experiments were performed. First, nine pigs were immunized by injection with the attenuated ASFV isolate E70-MS14, titers of anti-HA antibodies were estimated, and then the pigs were challenged with the homologous virulent isolate (E70). Table 4 shows the pigs ordered by their production of anti-HA antibodies ranging in titer from 20 to 5120. Only one of the nine pigs showed a significant index of infection–inhibition of 3 (the pig having the maximal titer of anti-HA antibodies) and was able to survive without detectable viremia to the homologous virulent challenge. Furthermore, no

TABLE 4

TITER OF ANTIBODIES IN SERUM FROM PIGS IMMUNIZED WITH E70-MS14 AND DAYS OF VIREMIA AFTER CHALLENGE WITH E70

Pig No.	Titers after immunization			Viremia (days)
	iHag	iI	IF	
1322	5120	3	8000	0
1463	1280	0.6	4000	28
1653	80	0	800	21
1674	80	0	400	63
1661	20	0	1600	28
1652	80	0	800	14 (+)
1657	80	0	1600	16 (+)
1650	40	0	1600	12 (+)
1654	40	0	800	16 (+)
Control	0	0	0	7 (+)

Note. Pigs were injected with the sonicated cytoplasmic fraction of MS cells infected with attenuated E70-MS14 in Freund's adjuvant. One month after the last injection the pigs were first bled (titers after immunization) and then challenged by introducing an E70-infected pig and a control nonimmunized pig into the same room. After the challenge, the pigs had viremia (days) and then died (+) or survived. iHag, titer of inhibition of agglutination. iI, titer of infection–inhibition. IF, titer of anti-ASFV as measured by immunofluorescence.

chronical ASF lesions were found when this pig was sacrificed 6 months later. However, no correlation between anti-HA titers and survival after challenge was found because four pigs having low anti-HA titers died but also three pigs having low anti-HA titers survived.

Second, a study was made by following up the antibodies in the serum after the infection of a pig with the attenuated ASFV isolate E1207-VR11. Figure 4 shows that the titers of anti-Had and immunofluorescence followed a similar continously increasing trend to 80 days after infection. Parallel increasing profiles were obtained for the titers of anti-HA (measured by inhibition of Hag), infection–inhibition index, and anti-HA (measured by ELISA) until the end of the viremia (about 30 days after infection). Once the viremia was not detectable, all these titers remained constant, suggesting their dependence on viral replication.

Anti-HA antibodies in HA-injected pigs

To obtain a first estimation of the HA antigenicity, pigs were immunized with concentrated HA and its serum antibodies were examined periodically. Four intraperitoneal injections of 250,000 U each of HA mixed with Freund's adjuvant were given monthly and one injection of 1×10^6 HA U was given in the fifth month. A maximum titer of 2500 of anti-HA antibodies could be obtained with an injected optimal HA total dosage of 2×10^6 U. Further injections up to a total of 4×10^6 U of HA during 2 additional months did not increase the anti-HA titer (not shown).

An immunization protocol was then used with the optimal HA dosage inoculated as HA bound to Con A–Sepharose beads. Because of the difficulty of obtaining such large amounts of HA only two pigs could be immunized with a first intraperitoneal injection of 2×10^6 U of HA bound to 3 ml of Con A–Sepharose beads followed by a second injection 1 month later. One month after the second HA injection no anti-HA antibodies were detectable in the serum of either of the two HA-immunized pigs. After the pigs were challenged by cohabitation with an E1207-infected pig, one of them died of ASF and the other survived without showing either viremia or chronical ASF lesions but only fever. An immunoblot of the serum from the HA-immunized E1207-challenged and survivor pig against ASFV-infected MS cell extracts showed only two bands (at 51 and 54 kDa) not found in the immunoblot of the serum from the control nonimmunized pig. However the 54-kDa band also appeared when immunoblot of the HA-immunized pig serum was made against noninfected MS cell extracts (not shown). Also, a faint 51-kDa band but no 54-kDa band was found in the immunoblot of serum from the E1207-VR11-immunized E1207-challenged and survivor pig (Fig. 5).

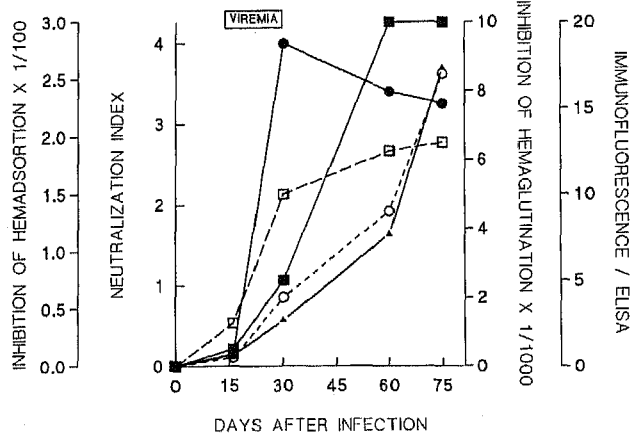

FIG. 4. Titers of inhibition of hemadsorption (Had), hemagglutination (Hag) and infection, and titers of immunofluorescence and of ELISA of sera from a pig infected with the E1207-VR11 isolate. The pig was infected with ASFV by intramuscular injection of 10^6 $TCID_{50}$ of isolate E1207-VR11. Bleedings were made periodically at the times indicated in *X*-axis and titers and viremia were obtained. (▲) Titer of inhibition of Had; (■) titer of inhibition of HA; (●) infection–inhibition index; (○) titer of immunofluorescence; (□) titer of ELISA.

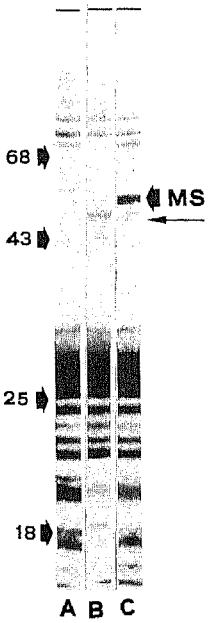

FIG. 5. Immunoblotting of pig sera against ASFV-infected MS extracts. Lane A, control nonimmunized pig; lane B, pig inoculated with ASFV isolate E1207-VR11 and surviving challenge with virulent E1207; lane C, pig immunized with purified HA and surviving challenge with virulent E1207. Numbers to the left are the molecular weight markers run in parallel. MS (broad arrow to the right), protein band appearing also in immunoblottings against noninfected MS cell extracts. The thin arrow to the right indicates the position of the 51-kDa protein.

DISCUSSION

This work describes the isolation, purification, and characterization of a soluble ASFV-induced molecule

released into the cell culture medium in cells infected with some ASFV isolates causing hemagglutination of pig erythrocytes.

That most of the HA was free from complete particles of ASFV was demonstrated by its 95% recovery after removal of most of the viral particles by ultracentrifugation, by the low Hag activity recovered in the ASFV-containing pellet, and by the separation of the detectable HA and the ASFV particles by gel-permeation chromatography. A 4.7–6.9% of the initial Hag was detected in the ultracentrifugation pellet when using large amounts of starting HA (millions of units), but this Hag activity could be due to contamination with host membranes (Carrascosa *et al.*, 1985) rather than to the presence of HA in the ASFV particles. When low amounts of HA (Fig. 2) or low-ionic-strength buffers (preliminarly experiments) were used for the Hag assay, the Hag activity was not detected in the ASFV.

The 300-kDa molecular weight estimation for the native HA by gel-permeation chromatography (Fig. 2) and that of 51 kDa for denatured HA by immunoblotting (Fig. 5) together with the HA sensitivity toward denaturing protein agents and its carbohydrate-containing behavior (Table 2) suggest that the HA molecule induced by ASFV is a complex of 300 kDa made of 51-kDa glycoprotein(s) monomers.

The presence of cellular and viral protein complex patterns in the 51-kDa region was demonstrated in E70- and E608-infected pig macrophages (Alcaraz *et al.*, 1992). Similarly the presence of actin (45 kDa), β-tubulin (56 kDa), α-tubulin (58 kDa), 50- and 54-kDa cellular proteins in addition to the 50- and 54-kDa viral-induced proteins has also been demonstrated by two-dimensional electrophoresis of ^{35}S-labeled proteins of ASFV-infected VR cells (Esteves *et al.*, 1986). The 56 (core)- and a 51 (envelope)-kDa viral proteins were labeled with [^{3}H]sugars in ASFV-infected MS cells (Tabares *et al.*, 1983; Arzuza *et al.*, 1992) and with ^{125}I-chloramine in the membrane of infected macrophages (Alcaraz *et al.*, 1989). Finally, a 51-kDa protein has also been detected among other proteins by precipitation of ASFV-infected cell extracts with one of the lectins (Con A) which agglutinates free ASFV (Del Val, 1985). All these 51-kDa proteins may be the same molecule(s) that, although mostly soluble, is also present in the ASFV virion (pelletable HA) as occurs with the HA in other viruses (Noda *et al.*, 1988; Trybala *et al.*, 1980; Collins and Knight, 1978; Gerlier *et al.*, 1988; Payne and Norrby, 1976).

ASFV-infected VR cells gave a strong membrane fluorescence when stained with pig serum containing a high titer of anti-HA antibodies (not shown), suggesting some of the HA antigens to be localized in the membrane of infected cells where they could cause Had. Most probably the molecules causing Hag and Had are structurally related because (a) of the seven isolates studied, all were both Had- and Hag-negatives or -positives; (b) several layers of erythrocytes are formed during Had, suggesting erythrocyte–erythrocyte interactions (Carnero *et al.*, 1967; Larenandie *et al.*, 1987); and (c) that seems to be the case in other viruses studied (Itamura *et al.*, 1990). Further experimentation should clarify this point.

Although some of the results reported here suggested some relationship between anti-HA antibodies and infection–inhibition indexes (for instance, only pig 1322 having the highest anti-HA and il titers survived without viremia) (Table 4), no strong evidence showed that to be the case (four pigs having low anti-HA titers died but one pig having anti-HA of only 20 survived). It is unknown why all the pigs did not behave similarly. However, induction of pig survival without viremia would be necessary (to avoid the risk of generating carrier pigs) to obtain a useful vaccine against ASFV; therefore, the very preliminarly result obtained with pig 1322 (Table 4) may be of enough interest to merit further studies.

The absence of the 51-kDa band from the immunoblot of serum from the E1207-VR11- or the HA-immunized pigs before challenge (not shown) suggests the low antigenicity of the HA, confirmed by the need of ASFV challenge to detect any positive reaction (Fig. 5). Furthermore, repeated injections of three mice with Con A-purified HA during 6 months failed to produce a >80 titer of HA inhibition (not shown). Next, the HA should be cloned and expressed in a host capable of glycosilation in order to obtain the amounts needed to increase its antigenicity so that meaningful immunization/ASFV-protection experiments can be done to study the role of HA in immune protection, if any.

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