The African Swine Fever Virus Proteins p54 and p30 Are Involved in Two Distinct Steps of Virus Attachment and Both Contribute to the Antibody-Mediated Protective Immune Response

Paulino Gómez-Puertas, Fernando Rodríguez,[1] José M. Oviedo, Alejandro Brun,
Covadonga Alonso, and José M. Escribano[2]

Centro de Investigación en Sanidad Animal (CISA-INIA), Valdeolmos, 28130 Madrid, Spain

The nature of the initial interactions of African swine fever (ASF) virus with target cells is only partially known, and to date only the ASF virus protein p12 has been identified as a viral attachment protein. More recently, antibodies to viral proteins p54 and p30 have been shown to neutralize the virus, inhibiting virus binding and internalization, respectively. Therefore, we investigated the role of these proteins in the receptor-mediated ASF virus endocytosis in swine macrophages, the natural host cells. Proteins p54 and p30, released from ASF virus particles after treatment of virions with a nonionic detergent, bound to virus-sensitive alveolar pig macrophages. Binding of these proteins was found to be specifically inhibited by neutralizing antibodies obtained from a convalescent pig or from pigs immunized with recombinant p54 or p30 proteins. The baculovirus-expressed proteins p54 and p30 retained the same biological properties as the viral proteins, since they also bound specifically to these cells, and their binding was equally inhibited by neutralizing antibodies. Binding of ^{35}S-labeled recombinant p54 and p30 proteins to macrophages was specifically competed by an excess of unlabeled p54 and p30, respectively. However, cross-binding inhibition was not observed, suggesting the existence of two different saturable binding sites for these proteins in the susceptible cells. In addition, protein p54 blocked the specific binding of virus particles to the macrophage, while protein p30 blocked virus internalization. Both proteins independently prevented virus infection and in a dose-dependent manner, suggesting that binding interactions mediated by both proteins are necessary to give rise to a productive infection. The relevance of blockade of virus–cell interactions mediated by p54 and p30 in the protective immune response against ASF virus was then investigated. Immunization of pigs with either recombinant p54 or p30 proteins induced neutralizing antibodies which, as expected, inhibited virus attachment or internalization, respectively. However, immunized pigs were not protected against lethal infection and the disease course was not modified in these animals. In contrast, immunization with a combination of p54 and p30 proteins simultaneously stimulated both virus neutralizing mechanisms and modified drastically the disease course, rendering a variable degree of protection ranging from a delay in the onset of the disease to complete protection against virus infection. In conclusion, the above results strongly suggest that proteins p54 and p30 mediate specific interactions between ASF virus and cellular receptors and that simultaneous interference with these two interactions has a complementary effect in antibody-mediated protection. © 1998 Academic Press

INTRODUCTION

African swine fever virus (ASFV) is an enveloped icosahedral deoxyvirus responsible of a devastating disease of swine (Costa, 1990; Viñuela, 1985; Wilkinson, 1989). This virus is formed by a single molecule of DNA of about 170 kb which contains about 150 ORFs and shares several structural features with the DNA of poxviruses (Yáñez *et al.*, 1995). The virus infects domestic pigs and wild boars and multiplies in soft ticks, which can act as a natural reservoir and a way of transmission, rendering its control difficult (Wilkinson, 1989).

Studies about the immune response against this virus have demonstrated that neutralizing antibodies are induced in ASF convalescent pigs (Gómez-Puertas *et al.*,

1996; Ruiz-Gonzalvo *et al.*, 1986a; Zsak *et al.*, 1993). These antibodies are able to neutralize the virus before and after binding to susceptible cells, inhibiting both virus attachment and internalization (Gómez-Puertas *et al.*, 1996). At least three ASF virus structural proteins, p72, p54, and p30, are targets for this kind of antibody (Gómez-Puertas *et al.*, 1996; Zsak *et al.*, 1993). Antibodies to proteins p72 and p54 inhibit a first step of the viral replication cycle related to virus attachment, while antibodies to protein p30 are implicated in the inhibition of virus internalization (Gómez-Puertas *et al.*, 1996).

A better knowledge of ASF virus–cell early interactions (for review see Angulo *et al.*, 1993a) is of great importance for ASF virus vaccine development. Morphological data obtained using electron microscopy have shown that ASF virus enters susceptible Vero cells and swine macrophages by endocytosis (Alcamí *et al.*, 1989, 1990; Geraldes and Valdeira, 1985). Virus particles have been found adsorbed to cytoplasmic invaginations similar to the clathrin-coated pits, which are involved in the entry of

ligands through a receptor-mediated endocytosis mechanism (Pearse, 1987). Binding of ASF virus to one or several specific cellular receptors seems to be essential for a productive infection. Experiments to analyze the entry of the virus through nonspecific receptors demonstrated that ASF virus does not infect swine macrophages through Fc receptors and, therefore, that antibody-dependent virus entry into the cell is not a mechanism that facilitates the progression of ASF virus infection (Alcamí and Viñuela, 1991). The presence of cellular receptors that mediate ASF virus binding to target cells precludes the existence of one or several viral attachment proteins in the viral particle (Angulo et al., 1993a). Octylglucoside treatment of purified virus particles releases several proteins, from which only p12 binds specifically to virus-sensitive cells (Carrascosa et al., 1991). This protein competes with the binding of ASF virus to cells and reduces viral infectivity (Carrascosa et al., 1991; Angulo et al., 1993b). To date, p12 is the only characterized ASF virus protein involved in virus attachment to the host cell. However, pigs immunized with recombinant p12 develop neither protective immunity (Carrascosa et al., 1995) nor neutralizing antibodies (Angulo et al., 1993b; Gómez-Puertas et al., 1996).

At present, there is not an available vaccine against ASF, and only experimentally is it possible to confer solid protection to pigs against homologous virus challenge, by inoculation of attenuated viruses obtained by cell culture passage (Carrascosa et al., 1995; Escribano et al., 1993; Zsak et al., 1993). These attenuated viruses induce in vaccinated pigs antibodies that neutralize the virus infectivity in cell lines and pig macrophage cultures (Gómez-Puertas et al., 1996; Gómez-Puertas and Escribano, 1997; Ruiz-Gonzalvo et al., 1986a; Zsak et al., 1993). However, the relevance of such neutralizing antibodies raised in these experimental models in protection is poorly understood, and the identities of viral antigens capable of inducing the protective response are largely unknown. Previous scientific reports concerning the role of antibodies in ASF virus protection showed that when these antibodies were transferred through colostrum they conferred to suckling piglets a degree of protection against virus challenge (Schlafer et al., 1984a,b). Passive transfer experiments using anti-ASFV immunoglobulins indicate that ASFV antibodies alone protect pigs against lethal infection (Onisk et al., 1994). These experiments also suggested that this antibody-mediated protective effect involves a critical early event that delays disease onset.

p30 and p54 are externally located virus proteins (Gómez-Puertas et al., 1996; Rodríguez et al., 1996) of 30 and 25 kDa, encoded by the virus genes CP204L and E183L, respectively (Afonso et al., 1992; Rodríguez et al., 1994; Yáñez et al., 1995). These proteins have previously been expressed in a baculovirus system for neutralization studies (Gómez-Puertas et al., 1996) and used in diag-

nostic tests due to their antigenicity (Oviedo et al., 1997). In the present study, we have investigated the role of the neutralizing proteins p30 and p54 in the early stages of ASF virus infection and their role in the protective immune response.

RESULTS

Binding of ASF virus proteins to macrophages

External ASF virus proteins were released from viral particles by detergent treatment of purified virions. The solubilized proteins with the different treatments were those previously described (Tabarés et al., 1980, 1981; Carrascosa et al., 1991; not shown). From virus proteins solubilized with OG, only protein p12, the previously described ASF virus attachment protein, bound specifically to macrophages (Fig. 1A). However, when virus external proteins were solubilized with NP-40 detergent, two additional proteins of 25 and 30 kDa were detected as attached to macrophages (Fig. 1A). The use of 0.5 M NaCl in the treatments did not modify the number of solubilized proteins attached to susceptible cells. Similar experiments of incubation with virus-released proteins were carried out in the presence of specific neutralizing anti-p30, -p54, or -ASF virus sera. As expected, serum against protein p30 inhibited specifically cell binding of the viral protein with an electrophoretic mobility of 30 kDa. Similarly, serum anti-p54 inhibited only the binding of the 25-kDa viral protein (Fig. 1A). Hyperimmune serum anti-whole virus inhibited the binding of all viral proteins to macrophages, including protein p12. These data of specific binding inhibition by monospecific antisera pointed out that the structural proteins attached to the cell correspond to proteins p30 and p54.

To further confirm the identity of the 25- and 30-kDa proteins that bind to susceptible cells, we radiolabeled the proteins induced by recombinant baculoviruses expressing p54 (Bacp54) and p30 (Bacp30) with [^{35}S]Met/Cys. The resulting patterns of labeled proteins after infection with the recombinants are shown in Fig. 1B. Recombinant proteins p54 and p30 were immunolocalized by a Western blot using monospecific antisera (data not shown). Then, labeled recombinant proteins were used for binding experiments. These binding experiments were performed in the presence of a normal nonimmune pig serum or in the presence of anti-p54 or -p30 sera or hyperimmune serum. From the proteins contained in the insect cell extracts, only recombinant proteins p54 and p30 bound specifically to macrophages when negative serum was present during incubation (Fig. 1B). Specific antibodies inhibited the binding of these two recombinant proteins (Fig. 1B), as occurred with the binding of the same proteins released from the virus particles (Fig. 1A). Other recombinant proteins expressed in baculovirus (glycoprotein S of transmissible gastroenteritis virus or β-galactosidase) did not attach to macrophages (data not shown). These results confirm the identity of both
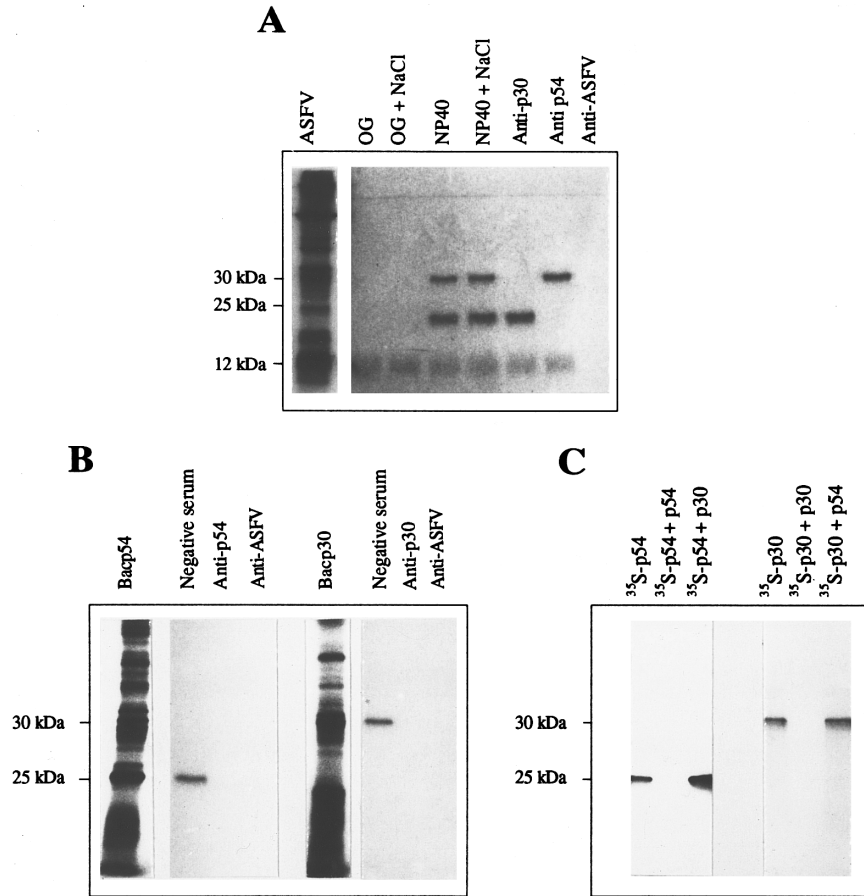
FIG. 1. Binding of ASF virus proteins to alveolar swine macrophages. (A) Proteins in the whole ^{35}S-labeled virus purified by Percoll sedimentation (ASFV), and proteins released from virions after different treatments with OG and NP-40 in the presence or the absence of NaCl, that bound to macrophages. Specific antibodies (Anti-p30, -p54, -ASFV), present during incubation of virus-released proteins, inhibited the binding of these proteins to macrophages. (B) Proteins labeled with [^{35}S]Met/Cys in the whole extracts from insect cells infected with recombinant baculoviruses expressing p54 (Bacp54) or p30 (Bacp30). This panel shows the specific binding to macrophages of the ASF virus proteins expressed in this system in the presence of nonimmune serum (negative serum) or sera against p54 or p30 (Anti-p54, -p30). (C) Determination of the existence of two different receptors for p54 and p30 by cross binding competition experiments of baculovirus-expressed p54 or p30. ^{35}S-labeled p54 (^{35}S-p54) or p30 (^{35}S-p30) were incubated with macrophages individually or simultaneously with an excess (80:1) of unlabeled proteins (p54 or p30).

virus proteins and illustrate how these baculovirus-expressed proteins retain the biological properties of the protein naturally incorporated into virions.

X-ray films showing protein patterns of purified virus (Fig. 1A) or baculovirus extracts containing the recombinant p30 or p54 (Fig. 1B) were exposed half time to that showing virus proteins attached to macrophages. Overexposure (four times more) of films showed a small background of proteins attached to macrophages composed of discrete bands corresponding to baculovirus-induced or cellular proteins whose intensity corresponded to their relative abundance in the infected cell extracts (data not shown).

In order to determine if both proteins p54 and p30 bind to the same cellular ligand, we carried out competitive binding experiments under the above described conditions. Cells were preincubated with an excess of unlabeled protein extracts (80:1), followed by incubation with labeled extracts. Binding of labeled protein was com-

pletely inhibited by an excess of the same unlabeled protein (Fig. 1C), suggesting that cell binding sites are saturable. No cross-binding inhibition between both proteins was observed under these conditions, implying the existence of two different binding sites for these proteins in the macrophage.

Inhibition of African swine fever virus infectivity by proteins p30 and p54

We studied the possible reduction on ASF virus infectivity produced by competition between recombinant proteins and virus for the same ligand in macrophages. To carry out these experiments we analyzed the virus infectivity reduction using different amounts of recombinant protein p30 or p54, together with other irrelevant proteins produced in the same baculovirus expression system which were used as controls. In the assayed conditions, proteins p54 and p30 produced an inhibition of ASF virus infectivity in a dose-
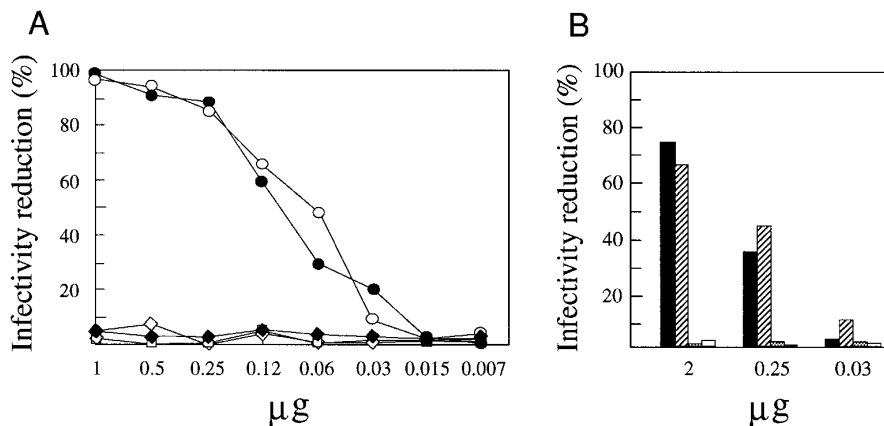
FIG. 2. Inhibition of alveolar swine macrophages infection by recombinant ASF virus proteins p54 and p30. (A) Different concentrations of competitor recombinant proteins p30 (open circles), p54 (solid circles), bcl2-like ASF virus protein (open squares), glycoprotein S of transmissible gastroenteritis virus (solid rhombus), or wild-type baculovirus-infected cells extracts (open rhombus) were inoculated simultaneously with 1207VRΔTKβ-gal virus on macrophage cultures. The infected macrophages were visualized by X-gal staining, and the percentage of infectivity reduction was determined by comparing the number of infected macrophages in the absence of recombinant proteins. Results represent the means of three independent experiments, with standard errors lower than 9% in all cases. (B) Infectivity reduction assay for ASF virus by capping of virus cell receptors specific for p54 or p30. Three different concentrations of recombinant protein p54 (black bars), p30 (dashed bars), bcl2-like protein (doted bars), or wild-type baculovirus proteins (open bars) were used to coat microtiter plates where macrophages were cultured and infected by the 1207VRΔTKβ-gal virus. Infected macrophages were visualized as described above. Results represent the means of infectivity reduction data obtained from two independent experiments and referred to the number of PFU observed in microtiter plate wells not previously absorbed with recombinant proteins. Standard errors were lower than 10% in all cases.

dependent manner (Fig. 2A). Other recombinant or wild-type baculovirus proteins had no effect on virus infectivity (Fig. 2A). No differences between p54 and p30 were observed with respect to the infection inhibition percentages obtained at the same protein concentrations. About 100% inhibition of virus infectivity was observed with 1 μg of both recombinant proteins.

To confirm the above results, we carried out infectivity reduction experiments in cells cultured on microplates previously absorbed with different concentrations of relevant or irrelevant recombinant proteins and wild-type baculovirus extracts. Under those conditions, capping of cell receptors for these proteins might occur, diminishing the probabilities of ASF virus binding to the cells and a successful productive infection. Proteins p54 and p30 produced an efficient capping of receptors as deduced by the observed inhibition of ASF virus infectivity in a dose-dependent manner (Fig. 2B). Other recombinant or wild-type baculovirus proteins again did not interfere with the virus infection (Fig. 2B).

Role of the ASF virus attachment proteins p30 and p54 during the first stages of infection cycle

To investigate the role of ASF virus proteins p30 and p54 during viral infection, radiolabeled purified extracellular ASF virus particles were used. Recombinant p54 or p30 proteins interfered with replication of this labeled and purified virus, while wild-type baculovirus or an irrelevant recombinant protein did not affect virus infectivity (Fig. 3A). Then, binding competition experiments in alveolar macrophages were performed. The results of

this study demonstrated that virus–cell binding reached a plateau 120 to 240 min after the reaction was started, as previously described (Gómez-Puertas et al., 1996). The presence of recombinant p54 in the cultures inhibited about 60% of virus attachment to macrophages at the time of maximum binding in the absence of this protein (Fig. 3B). In contrast, the recombinant protein p30 did not interfere with the virus attachment to cells, as found with other baculovirus extracts used as controls (Fig. 3B). These data suggest that protein p54 is implicated in a first step of the infection cycle related to virus–cell attachment.

To analyze the role of protein p30, we tested inhibition of labeled virus internalization in the presence of this protein. Under the conditions used, internalization of the virus previously attached to the cells was affected by addition of recombinant p30. More than 90% of the virus was released from the cells by protease treatment 4 h after the temperature of the cells was reversed from 4 to 37°C in the presence of p30 (Fig. 3C). Control proteins expressed in baculovirus or recombinant p54 did not interfere with normal virus internalization (Fig. 3C). These results strongly suggest that the binding of protein p30 to its cell receptor might constitute a second step of ASF virus replication cycle related to virus internalization.

Antibody response in immunized pigs against recombinant p54 and p30 ASF virus proteins

To analyze the immune response induced by proteins p54 and p30 involved in ASF virus–cell interactions, different groups of pigs were immunized with the recombi-
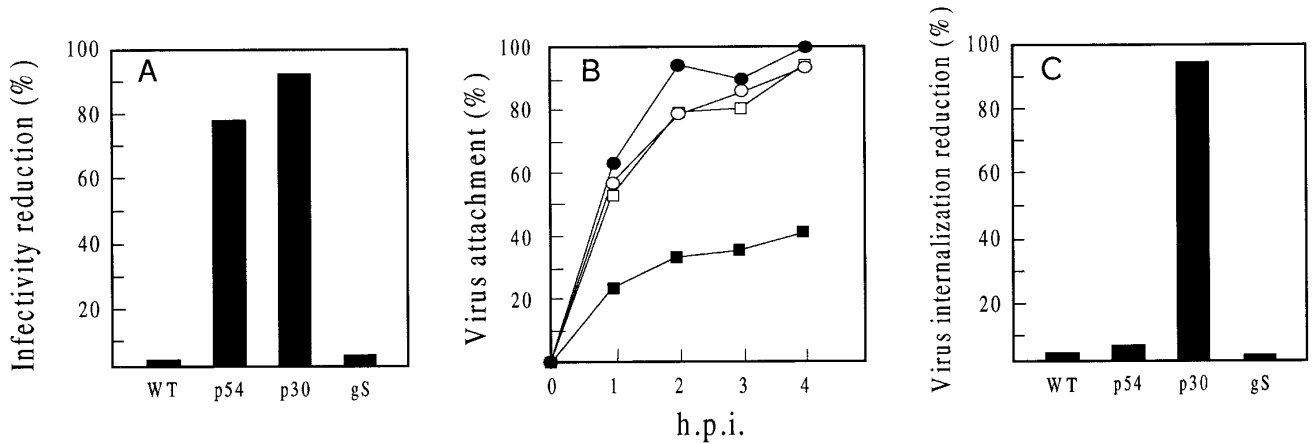
FIG. 3. Correlation between ASF virus infectivity reduction by competitor proteins and virus attachment and internalization inhibitions by the same proteins. (A) Infectivity reduction of 100 PFU of the highly purified ^{35}S-labeled 1207VRΔTKβ-gal virus produced by 0.4 μg of the baculovirus-expressed p54, p30, glycoprotein S of transmissible gastroenteritis virus, or wild-type baculovirus proteins. Results represent the means of infectivity reduction data obtained from two independent experiments and referred to the number of PFU observed in the absence of recombinant proteins. Standard errors were lower than 4% in all cases. (B) Percentage of ^{35}S-labeled virus bound to swine macrophages at different times postinfection at 4°C in the presence of 0.4 μg of competitor recombinant proteins p54 (solid squares), p30 (open circles), glycoprotein S (open squares), or wild-type baculovirus proteins (solid circles). Results represent the means of data obtained from three independent experiments and referred to the percentage of the virus bound in absence of recombinant proteins. Standard errors were lower than 9% in all cases. (C) Percentage of internalization reduction of ^{35}S-labeled virus into macrophages in the presence of 0.4 μg of competitor recombinant proteins p54 or p30, glycoprotein S, or wild-type baculovirus proteins. Results represent the means of data obtained from three independent experiments and refer to the percentage of the virus internalized in the absence of recombinant proteins. Standard errors were lower than 6% in all cases.

nant proteins p54 or p30 as well as a combination of these two proteins. Three pigs (6–8) were immunized with protein p30, three pigs (9–11) with protein p54, and six pigs (12–17) were vaccinated with p30 and p54 proteins simultaneously. Pigs were bled 15 days after the last inoculation, and a Western blot analysis showed that the antisera reacted against the proteins used for immunization (Fig. 4A). Antisera were also analyzed in a neutralization assay in pig macrophages, showing neutraliz-

ing titers, expressed as the maximum serum dilution that neutralized 50% of virus infectivity, between 1:50 to 1:200 (Fig. 4B). Four additional pigs (18–21), infected orally with the attenuated virus isolate E75CV1-4, were used to analyze the neutralizing antibody induction in comparison to pigs immunized with recombinant proteins. The neutralization titers raised in these pigs were similar to those found in sera from pigs immunized with recombinant proteins (Fig. 4B).
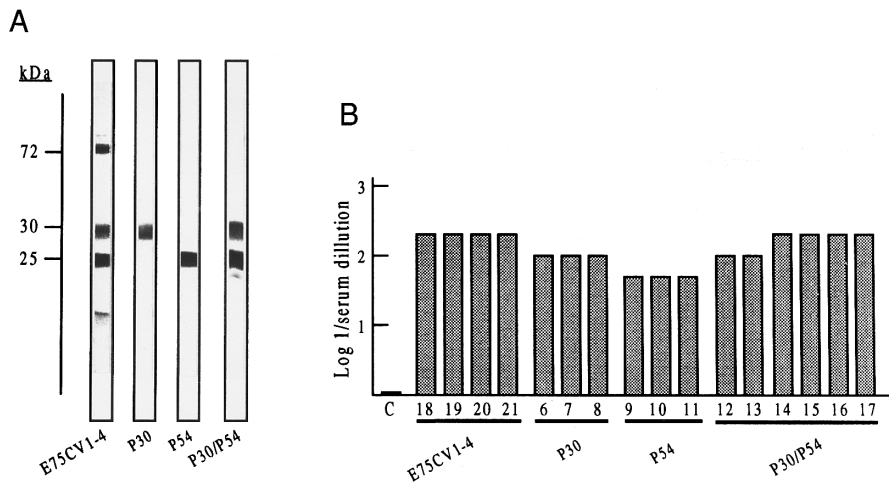

FIG. 4. Antibody response in different groups of vaccinated pigs. (A) Western blot showing the ASF virus proteins that react with representative sera from pigs infected with an attenuated virus (E75CV1-4) or immunized with recombinant ASF virus proteins p30 or p54 or a combination of these proteins. (B) Neutralizing titers of sera from the different groups of vaccinated pigs, plus a nonimmune serum (C) used as control. Results, expressed as means of three independent neutralization experiments, are indicated as logarithm of the maximum serum dilution that neutralized 50% of virus infectivity in pig macrophages.

In order to elucidate differences in neutralization that could determine protection or disease course modifications in vaccinated pigs, we studied the neutralization mechanisms elicited after immunization with different proteins. Immunization of pigs with p54 rendered antibodies that neutralized the virus only before attachment to the cell. In contrast, antibodies raised in pigs immunized with protein p30 were able to neutralize the virus before and after virus binding to the cells. Pigs vaccinated with the two recombinant proteins or with the attenuated virus E75CV1-4 neutralized the virus before and after binding to the cells (data not shown).

Antibody binding inhibition experiments with radiolabeled virus were carried out using the different sera and showed that pigs immunized with p54 inhibited ASF virus binding to susceptible cells. In contrast, antibodies induced in pigs immunized only with p30 did not inhibit this step of virus infection, but avoided virus internalization. Pigs vaccinated simultaneously with recombinant proteins p54 and p30 developed antibodies that inhibited both virus binding and internalization, similarly to antibodies from pigs vaccinated with the attenuated virus E75CV1-4 (data not shown).

Protective immunity conferred by proteins p30 and p54 in vaccinated pigs

At day 15 after the last immunization with recombinant proteins, all pigs were challenged intramuscularly with the highly virulent virus isolate E75. At 45 days postinoculation with the attenuated virus E75CV1-4, recovered animals were also challenged with the same dose of the same virus. Clinical signs of ASF such as fever, anorexia, lethargy, recumbency, and cyanosis were monitored daily.

Mortality among control group animals ($n = 5$) was 100% on postinoculation days 5 or 6 (Fig. 5A). In contrast, solid protective immunity to E75 challenge was observed in all E75CV1-4 recovered pigs (Fig. 5A). Animals remained clinically normal following challenge, with no fever or detectable viremia (Figs. 5B and 5C). Immunization of pigs with individual proteins (pigs 6–11) did not rendered any degree of protection to virulent virus challenge (Fig. 5A). Animals died between days 5 and 10 postinoculation. Comparative viremia titers and disease onset between control pigs and pigs immunized with individual recombinant proteins showed no significant differences (data not shown).

Interestingly, pigs immunized with a combination of p54 and p30 (pigs 12–17) presented dramatic changes in the disease course with respect to control pigs. After challenge, pigs 12 and 13 presented a delay in the onset of clinical signs of about 3 days (Fig. 5B), surviving to lethal infection after a transient symptomatic period. These pigs showed a reduction in maximum viremia titers with respect to control pigs (Fig. 5C) and remained
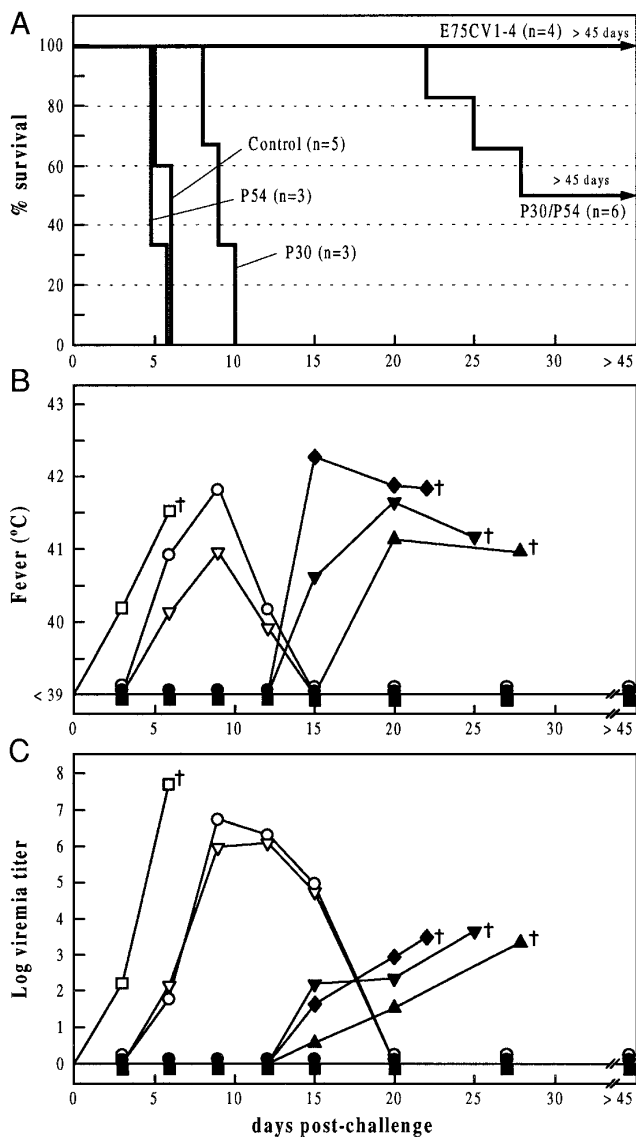

FIG. 5. (A) Effect of different vaccination procedures with the attenuated virus E75CV1-4 or recombinant proteins on pig survival following challenge with virulent ASF virus E75 (5×10^2 TCID$_{50}$), compared to unvaccinated control pigs. Fever (B) and viremia (C) titers in a representative control pig (□), in pigs vaccinated with the attenuated virus E75CV1-4 (■), and in pigs vaccinated with p30 and p54 (12:○, 13:▽, 14:▲, 15:◆, 16:▼, 17:●).

clinically normal during a postchallenge monitoring period exceeding 45 days. Three other pigs (14–16) immunized with these two proteins showed a longer delay in disease onset (more than 10 days than in animals immunized with individual proteins). Moreover, one animal (pig 17) remained asymptomatic during the postchallenge monitoring period (Fig. 5B). Viremia titers in pigs 14–16 were lower than those in pigs 12 and 13, increasing the virus titers more gradually along postchallenge days in these three pigs (Fig. 5C). After day 15 postchallenge, disease signs progressed to death in these three pigs. Viremia was not detectable at any time in pig 17, in which clinical signs were not observed (Fig. 5C).

The disease pattern observed in partially protected pigs was very similar to that observed in control pigs. The main difference was the duration of illness and the clinical evolution. Whereas animals belonging to the control group dead in about 2–3 days after clinical onset of the disease, immunized animals (pigs 12 to 17) evolved to resolution or death after a prolonged disease of about 10–12 days duration, in which pigs presented mainly fever, recumbency, and anorexia, with the exception of pig 17. This asymptomatic animal, after challenge inoculation, was farmed in close contact with pigs 14–16 during the postchallenge period, which also means direct contact with the virus during a long period of time and presumably multiple possibilities of reinfection. It suggests the induction of a solid protective immunity in this pig, similar to that observed after vaccination of pigs with attenuated viruses.

To ascertain the complete elimination of the virus in protected pigs after immunization with recombinant proteins (pigs 12, 13, and 17), pigs were euthanized between 46 and 50 days postchallenge inoculation, and the presence of virus in different organs (blood, spleen, lung, kidney, tonsil and maxillary, para-aortic, mesenteric, and mediastinal lymph nodes) was investigated by PCR. At necropsy no protected pigs showed characteristic ASF lesions in organs, with the exception of an enlarged maxillary lymph node from pig 13. Virus detection by PCR was carried out by using three different pairs of primers specific for amplification of p72, p54, and p30 virus genes. All systematically sampled organs were negative, with the exception of the above mentioned lymph node, in which PCR reactions were positive for the three analyzed genes (data not shown).

Finally, in pigs immunized with p30 and p54 that showed partial protection from virus challenge (pigs 14–16), we studied the possibility of *in vivo* generation of escape mutants to neutralization, which may represent a viral mechanisms to overcome the immune system. To demonstrate the presence of these escape mutants, viruses isolated at different postchallenge days were tested for neutralization by sera obtained before challenge. No differences in neutralization percentages were found between the virus used for the challenge and viruses recovered from viremic pigs (data not shown).

DISCUSSION

Several factors have limited progress in the design of a vaccine against ASF virus. One of the most important is the lack of knowledge of the precise interactions between the virus and a specific cellular receptor(s) and the entity of viral and cellular proteins implicated. Only one ASF virus protein, p12, has been previously characterized as involved in early interactions of the virus with susceptible cells (Carrascosa *et al.,* 1991). Our results strongly suggest the existence of two additional virus attachment proteins, p54 and p30, in addition to the previously described p12. The recombinant protein p12 expressed in baculovirus blocked the specific binding of virus particles to susceptible cells and prevented infection (Angulo *et al.,* 1993b; Carrascosa *et al.,* 1995), as we have demonstrated for proteins p54 and p30. Apparently, proteins p12 and p54 represent two ASF virus ligands with similar functions during the virus infection cycle, while p30 seems to act at a different step of the early virus–cell interactions. Another remarkable aspect of these attachment proteins is that while p54 and p30 have been correlated previously with the induction of neutralizing antibodies during infection (Gómez-Puertas *et al.,* 1996, 1997), antibodies specific for protein p12 did not either neutralize the virus infectivity *in vitro* nor protect pigs against ASF virus infection (Carrascosa *et al.,* 1995; Gómez-Puertas *et al.,* 1996). This feature of the immune response against the different ASF virus attachment proteins could be of great relevance in terms of future vaccine formulations.

Multiple virus attachment proteins could bind to different receptors or different binding sites of the same receptor, acting together either to modulate each other or to contribute in complementary functions. Alternatively, the receptors that bind to different virus ligands might act sequentially. Binding of the virus to the first receptor could cause changes in the virus or host that are necessary before the second receptor can bind (Spear, 1993). From our results, p30 and p54 seem to bind independently to their cellular receptors, since both proteins are able to bind individually to cells interfering with virus infectivity. Experiments with synergistic infectivity reduction by simultaneously adding both proteins did not render improved inhibition percentages over those with individual proteins (data not shown).

In the virus infection context, a first binding step of an attachment protein(s) to its cell receptor(s) (i.e., p12 or p54) could constitute a rapid binding, which could be followed by a slower or more difficult binding to a second receptor(s) (i.e., p30 receptor) influenced by several factors. This mechanism of interaction has been documented in the phenomenon of adhesion of lymphocytes to endothelium (Lawrence and Springer, 1991). Multivalent binding sites greatly increase the avidity of binding (Roitt, 1991). The binding of several viral attachment proteins of enveloped viruses to receptors could lead to more stable binding. The complete binding and internalization of the ASF virus could apparently be achieved only after a strong adhesion process consisting of a cascade of events that include the interactions of several virus proteins with their cell receptors. The details and the sequence of events during the first stages of ASF virus infection are not completely known. However, the data presented here clearly assign specific virus proteins to at least two distinguishable steps of the infection

cycle of the ASF virus related to binding and internalization.

As mentioned above, the knowledge of proteins involved in the early steps of the infection cycle is of great importance in vaccine development against any virus disease. Apparently, from the *in vitro* experiments presented here, it could be expected that interfering with either p30 or p54 binding to their specific receptors, it may be possible to inhibit virus infection. However, *in vivo* experiments have shown that pigs are able to resist a virulent virus challenge only when they are immunized with p54 and p30 simultaneously. These vaccinated pigs developed antibodies that neutralize the virus before and after attachment to cells. The conclusion is that to achieve *in vivo* protection it is necessary to interfere with the binding of at least two viral attachment proteins to their specific cell receptors. The necessity of generating antibodies that block both virus attachment and internalization to obtain protection could suggest that the virus may escape from the immune response generated to individual attachment proteins by the alternative route of other virus proteins that could also bind to specific cell receptors. All these facts encourage us to extend this study to other possible virus attachment proteins for an efficient vaccine development against ASF virus.

Although we cannot rule out that other non-antibody-related immune mechanisms could be induced in animals immunized with recombinant proteins as previously described by other authors (Bachmann *et al.,* 1994; Weidt *et al.,* 1994), protective results obtained in vaccinated pigs correlate to the antibody-mediated neutralization mechanisms developed. Only animals presenting antibodies capable of neutralizing virus attachment and internalization simultaneously showed a degree of protection or significant modifications of the disease course. At present we cannot explain immune response differences that determine survival to virulent infection in the group of pigs vaccinated with p54 and p30. It cannot be ruled out that individual susceptibilities to ASF virus may determine resistance differences between distinct pigs immunized by similar procedures. Viremia titers did not represent a predictive parameter of the prognostic outcome of the disease in immunized pigs as has been recently shown (Ramiro-Ibáñez *et al.,* 1995).

The ability of ASF virus to replicate efficiently in different cell lineages appears to be a critical factor in ASFV virulence (Ramiro-Ibáñez *et al.,* 1995, 1996). Antibodies raised to p30 and p54 viral proteins mediate interference with early viral replication events in susceptible cells inhibiting virus binding and internalization. *In vivo,* pigs immunized against these proteins significantly delayed ASF disease onset, probably interfering with the virus efficiency of replication. This suggests that antibodies against these proteins may be responsible for altering both disease course and final outcome, similarly to an-

tibodies from protected pigs in passive transfer experiments (Onisk *et al.,* 1994).

Although antibodies inhibiting two different steps of virus infection protected 50% of immunized pigs, this protection was qualitatively different from immunity observed in attenuated virus-vaccinated pigs (Escribano *et al.,* 1993). Following virulent virus challenge, attenuated ASF virus-inoculated pigs remained clinically normal, with no detectable viremias. Only one animal, vaccinated with p54 and p30 recombinant proteins (pig 17), showed such a solid protective response. In most cases, protected pigs showed transient clinical disease and moderate levels of viremia (always in a lower range than viremia titers found in control pigs). This level of protection is similar to that obtained in immunized pigs with the ASF virus hemagglutinin expressed in baculovirus (Ruiz-Gonzalvo *et al.,* 1996).

In conclusion, proteins p54 and p30 interact with two different receptors or binding sites on the ASF virus susceptible cells in addition to the previously characterized attachment protein p12. The simultaneous blockade of ASF virus interactions mediated by p30 and p54 proteins rendered significant modifications of disease course after virulent virus challenge of immunized animals. Therefore, we may asses that antibodies raised to ASF virus proteins implicated in neutralization may protect pigs against lethal virus infection, and the stimulation of different antibody-mediated virus neutralization mechanisms is important for this protective immune response. The identification of ASF virus antigen entities responsible for inducing these protective antibodies is of great relevance for vaccine development against this disease.

MATERIALS AND METHODS

Cells, viruses, and sera

Porcine alveolar macrophages were collected by alveolar lavage (Gómez-Puertas *et al.,* 1995) and kept frozen in liquid nitrogen until use. Macrophages were cultured 24 h prior to inoculation in RPMI 1640 medium supplemented with 20% heat-inactivated swine serum and 10% fetal bovine serum.

Two ASF viruses were used to inoculate pigs, the cell-adapted attenuated virus E75CV1-4 and the parental highly virulent virus E75L8. The ASF virus isolate 1207VR15 and a derivative recombinant virus expressing β-galactosidase chromogenic marker gene into the thymidine kinase locus of the viral genome (1207VRΔTKβ-gal) (Gómez-Puertas *et al.,* 1995) were used for *in vitro* experiments.

Sera against proteins p30 and p54 expressed by recombinant baculoviruses (Gómez-Puertas *et al.,* 1996) were obtained from pigs immunized as described (Gómez-Puertas *et al.,* 1996). Hyperimmune anti-ASF virus serum was obtained from a convalescent pig after inoc-

ulation with the attenuated virus 608VR13 (Gómez-Puertas et al., 1996; Gómez-Puertas and Escribano, 1997).

Binding of ASF virus proteins to macrophages

The ASF virus strain 1207VR15 was radiolabeled with [35S]Met/Cys (300 μCi/ml) in Vero cells cultured in roller bottles and purified by Percoll sedimentation as described previously (Carrascosa et al., 1985). Suspensions of purified labeled ASF virus (10^5 cpm) in PBS were incubated with 0.5% of the nonionic detergents octylglucoside (OG) and Nonidet P-40 (NP-40) in the presence or the absence of 0.5 M NaCl for 2 h at 4°C. After treatment with the detergent, samples were centrifuged at 200,000 g for 2 h. Supernatants containing the detergent-released virus proteins were incubated with alveolar swine macrophages (about 150,000 cells) for 3 h at 4°C in culture medium as previously described (Carrascosa et al., 1991). Cells were washed three times with PBS and lysed with 2% sodium dodecyl sulfate buffer for SDS–PAGE analysis.

Labeled insect cells (300 μCi/ml) infected with recombinant baculoviruses expressing p30 or p54 ASF virus proteins were recovered at 72 h postinfection, washed with PBS, and lysed by hypotonic treatment (Na_2CO_3/ $NaHCO_3$ buffer, pH 8.3). Cell debris were clarified by centrifugation and a 1:10 volume of 10× PBS was added. About 5 x 10^5 alveolar macrophages were incubated with the soluble recombinant proteins contained in the cell extracts (3 x 10^4 cpm) for 1 h at 4°C. Then, cells were washed exhaustively with PBS and lysed with 2% sodium dodecyl sulfate buffer for SDS–PAGE analysis.

Interference of ASF virus proteins with virus infectivity

To study the effect on ASF virus infectivity produced by competition between recombinant proteins and virus for the same receptor in macrophages, we used the recombinant virus 1207VRΔTKβ-gal. Using this virus, a quantitation of infected cells by chromogenic dye staining is possible, allowing the determination of infectivity reduction percentages, similarly to a conventional plaque-forming reduction assay (Gómez-Puertas et al., 1995). The quantitation of baculovirus-expressed proteins was carried out by comparison between different known amounts of bovine serum albumin and different dilutions of soluble recombinant proteins contained in extracts from insect cell infected with recombinant baculoviruses, both stained by Coomassie blue. Polyacrilamide gels after staining were submitted to a densitogram and the intensities of bands were compared and quantified.

About 10^5 alveolar macrophages were infected with 100 PFU of the ASF recombinant virus in the presence of variable concentrations of recombinant proteins. After 4 h of incubation, cells were washed twice and fresh medium was added. At 14 h postinfection, infected macrophages were revealed with X-gal as described (Gómez-Puertas et al., 1995). The infectivity reduction data were obtained from three independent experiments and referred to the total infected cells obtained in the absence of recombinant proteins.

In experiments with capping of cell receptors for p30 and p54, three different concentrations of baculovirus-expressed proteins were used to coat microtiter plates overnight at 4°C. Plates were then washed with PBS and 5 x 10^5 alveolar macrophages were added to each well. After 2 h at 37°C, cells were inoculated with 100 PFU of the ASF virus and maintained for virus adsorption for 2 h at 37°C. Cultures were washed, and at 14 h postinfection infected cells were revealed with X-gal. The infectivity reduction data were obtained from two independent experiments and referred to the total number of infected cells yielded by microtiter plate wells without previous recombinant protein adsorption.

Competition of proteins with virus binding and internalization

Binding competition experiments in alveolar macrophages (1.5 × 10^5 cells) were performed at 4°C in the presence of baculovirus-expressed ASF virus proteins (0.4 μg), wild-type baculovirus extracts, or an irrelevant recombinant protein, using about 100 PFU of the Percoll-purified ASF virus 1207VRΔTKβ-gal radiolabeled in Vero cells with [35S]Met/Cys (300 μCi/ml), corresponding to 3000 cpm. At selected times postinfection, the supernatant was discarded and cells were washed three times with fresh culture medium. Then 2% SDS buffer was added, and the solubilized fraction radioactivity was measured in a liquid scintillation counter.

Internalization of 35S-labeled ASF virus into macrophages was determined by detachment of noninternalized virus by proteinase K treatment (Gómez-Puertas et al., 1996; Nguyen et al., 1986). 35S-labeled virus (100 PFU) was inoculated on alveolar macrophage cultures for 2 h at 4°C, allowing virus attachment but not internalization (Alcamí et al., 1989). After washing with fresh medium, baculovirus-expressed proteins at the same concentration as in the previous experiments were added to the cells and incubated for 2 h at 4°C. After washing, cultures were then incubated for 4 h at 37°C, the time required for about 90% of ASF virus internalization during a normal replication cycle (Gómez-Puertas et al., 1996). To remove the unpenetrated virus, proteinase K (Boehringer) at a concentration of 2 μg/ml in culture medium was used. After incubation with the enzyme for 45 min at 4°C, the supernatant and the cellular fraction were transferred to scintillation vials and the radioactivity was counted.

Vaccination and challenge procedures

Different groups of large white pigs (3 months old) were immunized with the recombinant proteins p54 or p30 as well as combinations of these proteins. Pigs received three doses of antigen administered intramuscularly in the presence of Freunds adjuvant (complete in the first inoculation and incomplete in the second and third inoculations). Three pigs (6–8) were inoculated with protein p30 and three (9–11) with protein p54, both expressed by recombinant baculoviruses (5 x 10^7 baculovirus-infected sf cells containing about 100 μg of recombinant protein per dose). Another group of pigs (12–17) were vaccinated with a combination of p54 and p30 proteins (5 $\times$ 10^7 to 10^8 baculovirus-infected cells per recombinant protein). Five unvaccinated pigs (1–5) were used as controls. Additionally, four pigs (18–21) were infected orally (Onisk *et al.,* 1994) with the attenuated virus isolate E75CV1-4.

All pigs were challenged intramuscularly with 5 $\times$ 10^2 $TCID_{50}$ of the highly virulent virus isolate E75. This dose represents a challenge of between 50 and 500 LD_{100} (Onisk *et al.,* 1994). Blood samples were collected previously to virulent virus challenge and at different postchallenge days for viremia determination, titrating the virus in porcine alveolar macrophages by detection of infected cells using specific antibodies (Ruiz-Gonzalvo *et al.,* 1986b). Titers were calculated and expressed as $TCID_{50}$/ml (Schlafer *et al.,* 1984a).

The neutralization activity of the sera was measured in pig macrophage cell cultures essentially as described previously (Gómez-Puertas *et al.,* 1995, 1996). These neutralization data were obtained from three independent experiments and referred to a nonimmune serum. The analyses of neutralization mechanisms of ASF virus mediated by antibodies were carried out as described elsewhere (Gómez-Puertas *et al.,* 1996).

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