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Neutralizing Antibodies to Different Proteins of African Swine Fever Virus Inhibit both Virus Attachment and Internalization

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African swine fever virus induces in convalescent pigs antibodies that neutralized the virus before and after binding to susceptible cells, inhibiting both virus attachment and internalization. A further analysis of the neutralization mechanisms mediated by the different viral proteins showed that antibodies to proteins p72 and p54 are involved in the inhibition of a first step of the replication cycle related to virus attachment, while antibodies to protein p30 are implicated in the inhibition of virus internalization.

African swine fever virus (ASFV) produces an economically significant disease of swine that is endemic in many African countries and others in the Mediterranean area. This icosahedral deoxivirus is formed by a single molecule of DNA of about 170 kb which shares several structural features with the DNA of poxviruses (34). ASFV induces approximately 100 polypeptides in pig macrophages, the natural host cells (5), and about 40 of these polypeptides have been described as being incorporated into the viral particle (9, 14).

At present, it is accepted that nearly all viruses have neutralization sites and that only ASFV and Marburg and Ebola viruses lack the ability to be neutralized by virus-specific antibodies (13). Little work has been done on ASFV neutralization, presumably because early experiments were structured and interpreted on the basis of the hypothesis that neutralizing antibodies are not induced by this virus (18, 33). However, other authors have demonstrated that different isolates of ASFV are neutralized by convalescent swine immune sera and monoclonal antibodies (26, 27, 35). Authors who showed neutralization described a persistent fraction of nonneutralized virus of about 10%. This persistent fraction was not demonstrable by an infection inhibition test, in which swine immune sera inhibited the infection by the homologous and sometimes heterologous viruses in pig macrophages (27).

The relevance of neutralizing antibodies in protection is poorly understood. Previous reports concerning the role of antibodies in ASFV protection showed that when those antibodies were transferred through colostrum they conferred to suckling piglets a degree of protection against virus challenge (29, 30). Passive transfer experiments using anti-ASFV immunoglobulin indicate that ASFV antibodies alone protect pigs against lethal infection (22). These experiments also suggest that this antibody-mediated protective effect involves a critical early event that delays disease onset.

Characterization of ASFV neutralization mechanisms. To study neutralizing antibody induction during ASFV infection, three groups of pigs held separately and composed by two, two, and six animals were inoculated with the attenuated viruses 1207VR15, 608VR13 (intramuscularly with 10^6 PFU), and E75CV1-4 (orally with $10^{5.6}$ 50% tissue culture infectious doses), respectively. From the group of pigs inoculated with the virus E75CV1-4, two animals died during the first 2 weeks after inoculation, showing characteristic symptoms of acute ASFV infection. The surviving pigs inoculated with this isolate presented detectable viremia for at least 3 weeks. Pigs inoculated with the 1207VR15 and 608VR13 isolates showed neither viremia nor clinical symptoms.

Convalescent swine sera obtained at 3 to 4 weeks after infection from the above-mentioned groups of pigs were heat inactivated (56°, 30 min) and examined for virus neutralizing activity in a plaque reduction test essentially as described previously (16). All analyzed sera neutralized at 1:5 dilution the four low-passage-number viruses 608VR13, 1207VR15, E70MS14, and 646VR20 in Vero cells, by reducing their infectivity between 87 to 100%. These neutralization data were obtained from three independent experiments and referred to a nonimmune serum. There was no substantial variation between sera obtained by inoculation of the different attenuated viruses. Pig serum against transmissible gastroenteritis virus, used as a hyperimmune control, failed to demonstrate any ASFV neutralizing activity. The persistent fraction of nonneutralized virus (4 to 13%), observed with most sera, was previously found by other authors (28, 35), and it has been also found in other enveloped viruses (8, 19, 23).

The kinetics of the appearance of neutralizing antibodies in infected pigs was studied by using sera recovered at different days postinfection from two pigs inoculated with the virus isolate E75CV1-4. Neutralization of more than 50% of virus infectivity was obtained with sera from day 9 postinfection in the two pigs (data not shown). Neutralizing antibodies reached a plateau in these pigs at the end of the second week after infection (days 12 to 14).

To analyze the mechanisms of ASFV neutralization, we first studied the blocking activity of virus attachment to cells (Vero cells and pig macrophages) mediated by antibodies. We compared virus neutralization percentages yielded by adding the antibodies before or after virus attachment to cells at 4°C, a temperature at which virus internalization is inhibited (2). Experiments on pig macrophages were performed with a recombinant virus expressing the β-galactosidase chromogenic marker gene (1207VRΔTKβ-gal) in a 14-h assay as described previously (16). Figure 1A shows that similar neutralization percentages were obtained in both cases, suggesting that neutralizing antibodies could inhibit different steps of the infection cycle.

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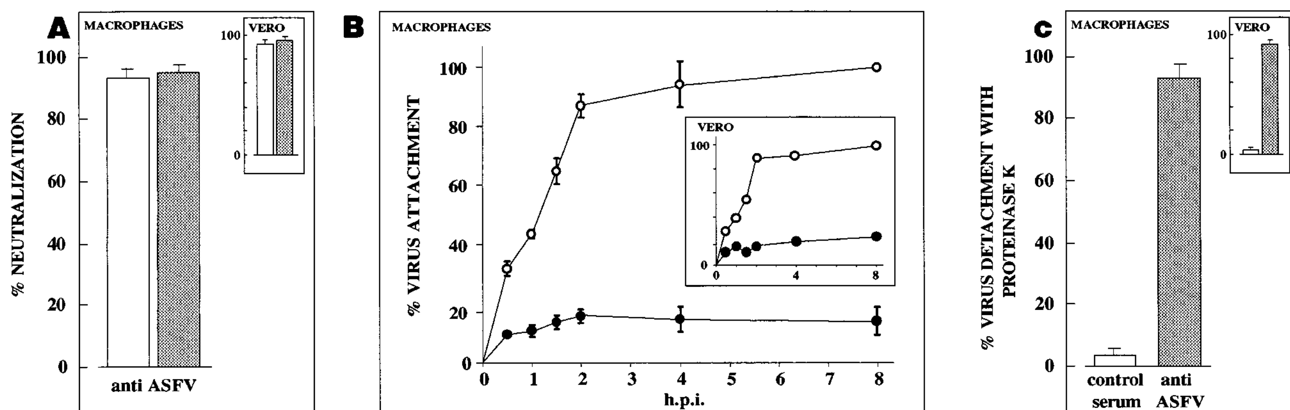

FIG. 1. Mechanisms of ASFV neutralization by convalescent pig sera. (A) Neutralization of ASFV before and after virus attachment to alveolar pig macrophages. Experiments were carried out with the 1207VRΔTKβ-gal recombinant strain of ASFV and a pool of anti-ASFV sera at 1:5 dilution. Open bar, neutralization percentages obtained when virus was preincubated with the serum before attachment to cells; gray bar, neutralization percentages obtained when antibodies were added after virus attachment for 2 h at 4°C. In both experiments, cells were washed and maintained for 14 h at 37°C. The infected macrophages were visualized by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) staining. The percentage of neutralization was determined by comparing the number of infected macrophages in the presence of the immune serum with that obtained in the presence of preimmune pig serum. The inset shows the results of similar experiments performed on Vero cell monolayers. Results represent the means ± standard errors (SE) of three independent experiments in both cases. (B) Binding inhibition experiments of ^{35}S-labeled ASFV, preincubated for 18 h at 37°C with a pool of anti-ASFV sera at 1:5 dilution (solid circles) or preimmune pig serum (open circles), layered on cultured pig macrophages or Vero cells (inset), and maintained at 4°C. The binding results, expressed as means ± SE of three independent experiments, are represented as percentages of the maximum attached radioactivity obtained (8 h postinfection in the presence of preimmune serum). (C) Internalization inhibition experiments of ^{35}S-labeled ASFV on Vero (inset) or alveolar macrophage cell cultures by using control preimmune serum (open bar) or the pool of anti-ASFV sera (gray bar) at 1:5 dilution. Unpenetrated virus was removed after 4 h at 37°C by proteinase K treatment. Results represents percentages of radioactivity recovered in the proteinase K supernatant fraction relative to the total supernatant-plus-cell-associated radioactivity and are expressed as means ± SE of three independent experiments.

Binding experiments with Vero cells or pig alveolar macrophages, using [^{35}S]Met/Cys-labeled virus at 4°C, showed that virus-cell binding reached a plateau 120 to 240 min after the reaction was started (Fig. 1B). A possible relationship between inhibition of virus binding to susceptible cells and virus neutralization was studied with three neutralizing convalescent swine sera. To carry out these experiments, 100 PFU of semipurified ^{35}S-labeled ASFV (corresponding approximately to 30,000 cpm) was mixed with immune or control swine sera at 1:5 dilution for 18 h at 37°C and inoculated on either Vero (5×10^4) or alveolar macrophage (5×10^5) cell cultures, and the cultures were maintained at 4°C. Macrophages were cultured in the presence of 30% nonimmune swine serum to avoid antibody-coated virus-Fc macrophage receptor interactions (4). At selected times postinfection, the supernatant was discarded and cells were washed three times with fresh culture medium. Then 50μl of 2% sodium dodecyl sulfate solution was added, and the solubilized fraction radioactivity was measured in a liquid scintillation counter. The results of this study demonstrated that binding of the virus to cells in the presence of anti-ASFV sera was inhibited more than 80% at the time of maximum binding in the absence of neutralizing antibodies, both in Vero cells and in macrophages (Fig. 1B). Parallel neutralization experiments demonstrated that those sera reduced radiolabeled virus infectivity more than 90%. These results confirmed the existence of an ASFV neutralization mechanism in which antibodies inhibit binding of the virus to cells.

To analyze the existence of a second ASFV neutralization mechanism, we tested inhibition of labeled virus internalization in the presence of immune sera. Internalization of ^{35}S-labeled ASFV into Vero cells or pig alveolar macrophages was determined by detachment of noninternalized virus by proteinase K treatment. ^{35}S-labeled ASFV (100 PFU) was inoculated on Vero or alveolar macrophage cell cultures for 2 h at 4°C, allowing virus attachment but not internalization (2). After washing with fresh medium, control or immune swine serum at 1:5 dilution was added to the cells, and the cultures were maintained for 2 h at 4°C. After washing, the cultures were incubated for 4 h, when about 90% of the virus was internalized during a normal replication cycle at 37°C in the absence of specific antibodies (data not shown). To remove the unpenetrated virus, proteinase K (Boehringer) at a concentration of 2 μg/ml in culture medium was used (21). 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. In those conditions, internalization of the virus previously attached to the cells was not affected by addition of a preimmune control serum (Fig. 1C). In contrast, 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 neutralizing sera (Fig. 1C). No significant differences were observed between the experiments performed with Vero cells and those performed with pig macrophages. These results revealed the existence of a second ASFV neutralization mechanism which abrogates a second step of the replication cycle involving virus internalization.

The results presented above demonstrated the existence of at least two mechanisms of ASFV neutralization in cells bearing specific receptors for the virus such as Vero cells and pig macrophages (2, 3). Virus binding and internalization assays were performed at a determined serum concentration. We cannot rule out that at different antibody-to-virus ratios, one of these mechanisms could be predominant as described for other virus models (32). However, under the conditions used in our experiments, the two mechanisms showed almost equivalent efficiency. About 80% of virus binding was inhibited and more than 90% of the virus was not internalized in the presence of neutralizing antibodies. The combination of both mechanisms neutralized more than 95% of virus infectivity. In summary, neutralization of ASFV may be the consequence of the inhibition of at least two steps of its replication cycle, virus attachment and internalization.

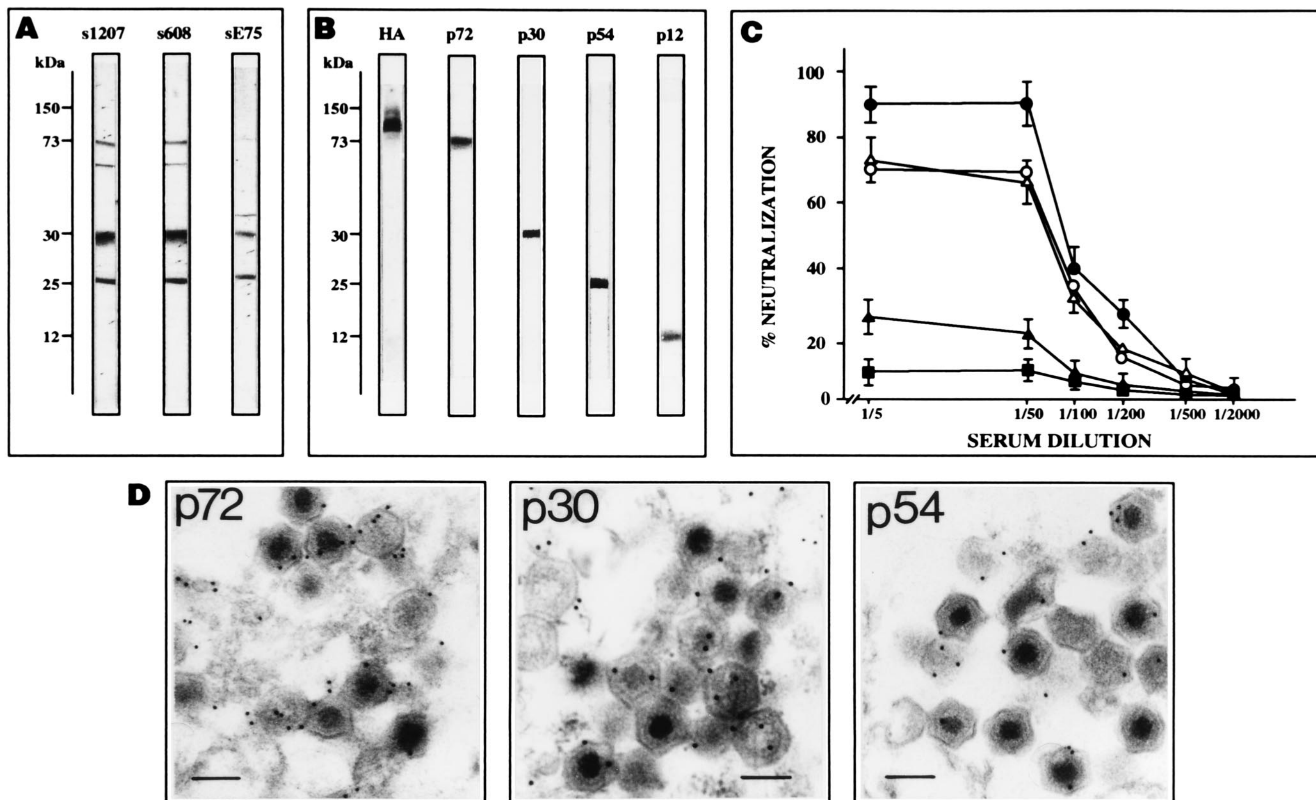

FIG. 2. Neutralization abilities of antisera to different viral proteins. (A) Western blot showing the ASFV proteins that react with sera from convalescent pigs infected with virus strains 1207VR15 (s1207), 608VR13 (s608), and E75CV1-4 (sE75). (B) Western blot showing the specificity of the monospecific pig sera raised to the recombinant ASFV proteins HA, p72, p30, p54, and p12. (C) Percentage of neutralization of the monospecific sera at different dilutions. The 1207VR15 strain of ASFV (100 PFU) was mixed with the anti-HA (solid squares), anti-p72 (open triangles), anti-p30 (solid circles), anti-p54 (open circles), and anti-p12 (solid triangles) monospecific sera at the dilutions indicated and subjected to a neutralization assay on Vero cells. Results, expressed as means ± standard errors of three independent experiments with all sera raised to the different proteins, are indicated as percentages of neutralization relative to a preimmune pig serum. (D) Electron micrographs of ASFV particles immunolabeled with antibodies to p72, p30, or p54. Ultrathin sections of formaldehyde-fixed 608VR13 virus-infected Vero cells at 24 h postinfection were labeled with an anti-p72, anti-p30, or anti-p54 monospecific antiserum and then with 10-nm protein A-gold. Bars, 200 nm.

Analysis of neutralizing antibody induction by individually expressed ASFV proteins. To our knowledge, p72 is the only ASFV protein described as being involved in virus neutralization (35). However, most viruses have more than one outer protein which mediates neutralization, and this contributes to the complexity of neutralization (13). Often each protein has multiple neutralization sites and probably different mechanisms of neutralization. Additionally, the efficiency of neutralization may vary between proteins.

A Western blot (immunoblot) analysis (6) of sera from convalescent pigs revealed that p72, p30, and p54 were three of the most antigenic proteins during infection (Fig. 2A), confirming previous reports (1, 6, 7, 24, 25). Therefore, we expressed these proteins in an *Escherichia coli* (p72) or baculovirus (p30 and p54) system. To express p72, a recombinant plasmid containing the p72 gene from the E70 ASFV strain, fused to the N-terminal fragment of MS2 polymerase under the control of the inducible $\lambda\ p_L$ promoter, was constructed by cloning the PCR-amplified ASFV gene, previously sequenced (20), into the pEX34 vector (31). The amplification was carried out with AmpliTaq DNA polymerase (Perkin-Elmer Cetus) and specific primers. The PCR products were digested with *Eco*RI and *Bam*HI and then cloned into the polylinker region of pEX34. The expressed p72-MS2 fusion protein was partially purified by sequential treatment with different concentrations of urea (31). DNA amplification of the p30 and p54 genes from the E70

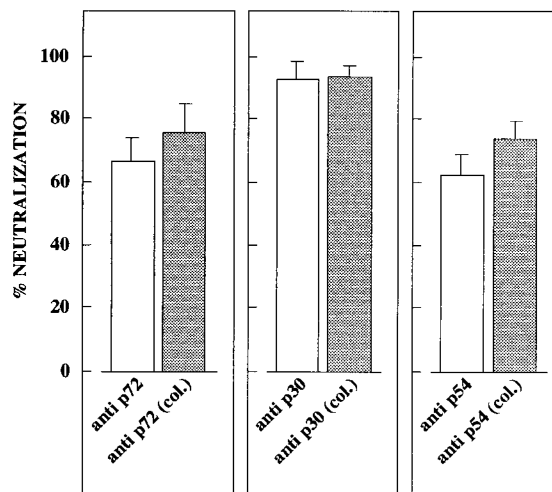

FIG. 3. Neutralizing antibodies generated to the different ASFV proteins during infection. Open bars, pool of monospecific antisera obtained from pigs immunized with the recombinant proteins; gray bars, affinity-purified antibodies to the different proteins obtained from the polyclonal antiserum s1207. All sera were diluted 1:5 and subjected to a neutralization assay on Vero cell monolayers. Results are expressed as means ± standard errors of three independent experiments. The experiment was also performed with affinity-purified antibodies from antiserum s608, and similar results were obtained.

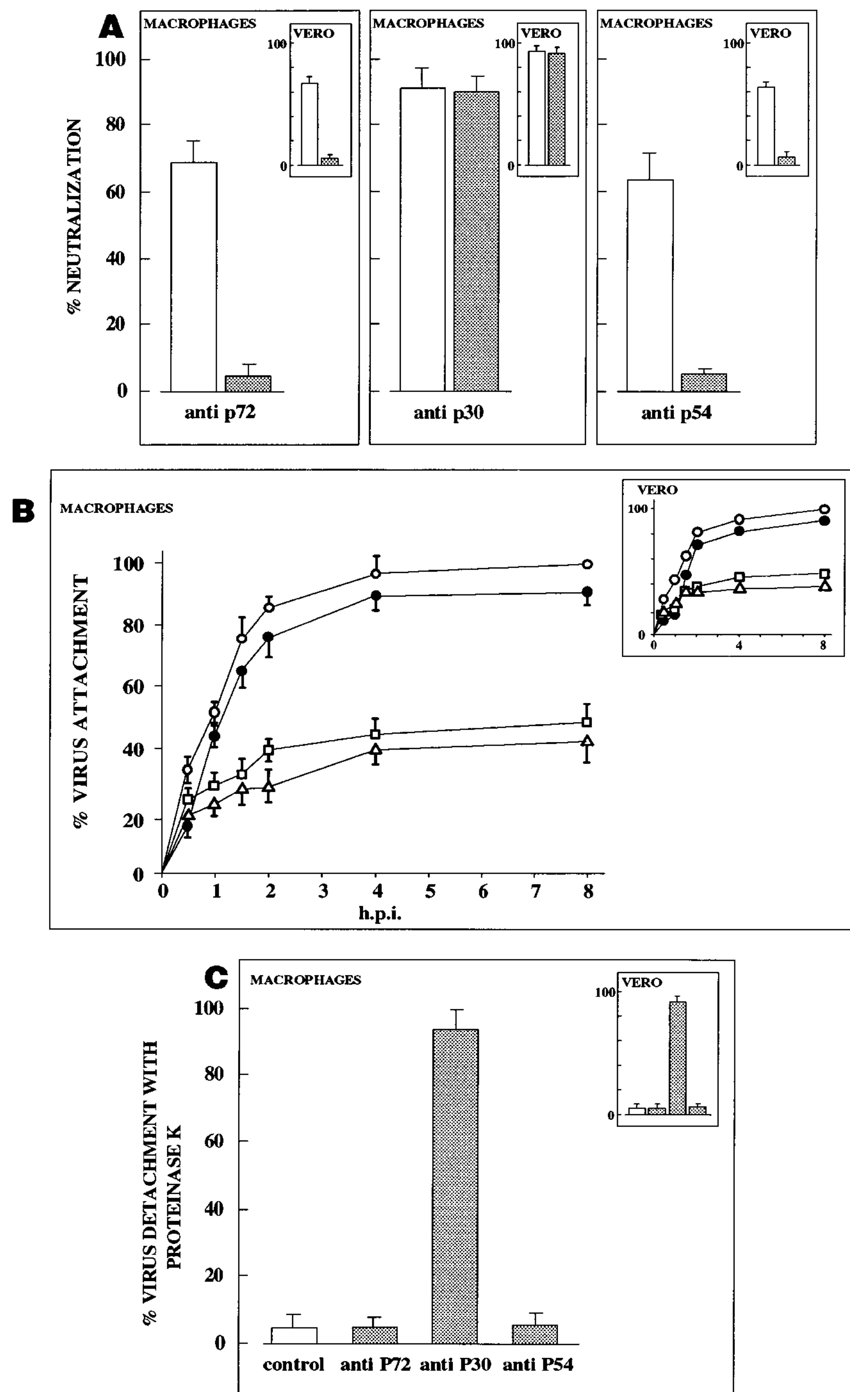

FIG. 4. ASFV mechanisms of neutralization mediated by different proteins. (A) Neutralization of ASFV (100 PFU of 1207VRΔTKβ-gal recombinant virus) before (open bars) and after (gray bars) virus attachment by monospecific anti-p72, -p30, and -p54 sera at 1:5 dilution. The infected macrophages were visualized by X-Gal staining. The percentage of neutralization was determined by comparing the number of infected macrophages in the presence of the immune serum with that obtained in the presence of preimmune pig serum. The insets show the results of similar experiments performed on Vero cells. Results represent the means ± standard error (SE) of three independent experiments in all cases. (B) Binding inhibition of ^{35}S-labeled ASFV preincubated with monospecific anti-p72 (open squares), anti-p30 (solid circles), and anti-p54 (open triangles) sera at 1:5 dilution or preimmune pig serum (open circles), layered on alveolar pig macrophages or Vero cells (inset), and maintained at 4°C. The results, expressed as means ± SE of three independent experiments, are represented as percentages of the maximum attached radioactivity obtained (8 h postinfection in the presence of preimmune serum). (C) Internalization inhibition experiments of ^{35}S-labeled ASFV on Vero (inset) or macrophage cell cultures by using control preimmune serum (open bar) or monospecific (gray bars) anti-p72, anti-p30, or anti-p54 sera at 1:5 dilution. Unpenetrated virus was removed after 4 h at 37°C by proteinase K treatment. Results represent percentages of radioactivity recovered in the proteinase K supernatant fraction relative to the total supernatant-plus-cell-associated radioactivity and are expressed as means ± SE of three independent experiments.

ASFV strain was carried out by PCR using specific primers. The PCR products were digested with the appropriate restriction enzymes and cloned into the pBakPAK 8 (Clontech) transfer vector under the control of the baculovirus polyhedrin promoter, which had been previously digested with the same enzymes. After sequence analysis of the cloned PCR products, recombinant baculoviruses expressing p30 or p54 were constructed and selected according to standard procedures. All recombinant proteins were analyzed for antigenicity by reactivity with the anti-ASFV sera or specific monoclonal antibodies (data not shown).

To raise high-titer anti-p72, -p30, and -p54 sera, pigs were immunized with the recombinant proteins. Two pigs received three inoculations of 250 μg each of the recombinant p72 administered intramuscularly in the presence of Freund's adjuvant (complete in the first inoculation and incomplete in the second and third inoculations). To obtain anti-p30 and -p54 sera, two pigs were intramuscularly inoculated with three doses of 2×10^8 baculovirus-infected Sf cells in the presence of Freund's adjuvant (complete for the first dose and incomplete for the others). All pigs were bled 15 days after the last inoculation, and Western blot analysis showed that the antisera obtained titers higher than 1:2,000 (Fig. 2B). Those antisera jointly with sera raised in pigs against the baculovirus-expressed viral attachment protein p12 (10, 11) or against the virus hemagglutinin (HA) (28), two or three sera, respectively, were analyzed in a neutralization assay. The results showed that only the sera against proteins p72, p30, and p54 neutralized more than 70% of virus infectivity at 1:5 dilution (Fig. 2C). Surprisingly, antisera raised against p12, the viral attachment protein, did not reduced virus infectivity in an in vitro assay. Furthermore, the ASFV HA, which confers a certain degree of protection against the virulent virus challenge to pigs immunized with the recombinant protein (28), induced antibodies that neutralized less than 30% of virus infectivity in a plaque reduction assay. Additionally, to confirm that the three neutralizing proteins were virus structural proteins, anti-p72, -p30, and -p54 sera were used in immunoelectron microscopy of ultrathin sections of ASFV-infected Vero cells as described previously (12). In this experiment, a multiplicity of infection of 10 was used, and the infected cells were collected at 24 h after infection. By this method, all three proteins were detected in mature intracellular viral particles (Fig. 2D).

To analyze the induction of neutralizing antibodies mediated by these three proteins in the context of infection, monospecific antibodies to p72, p30, and p54 were affinity purified (17) from two sera from pigs infected with the attenuated viruses 1207VR15 and 608VR13. The purified antibodies were lyophilized and resuspended in preimmune pig serum to the original volume. Then their specificity was confirmed by Western blotting, and they were used to carry out neutralization assays. Apparently, specific antibodies against proteins p72 and p54 generated during infection neutralized about 10% more virus than those obtained from the pigs immunized with the recombinant proteins (Fig. 3). No differences were found between antibodies against p30 obtained by affinity chromatography and those obtained by pig immunization with the baculovirus-expressed protein.

Characterization of the ASFV proteins implicated in the different neutralization mechanisms. The roles of different ASFV neutralization proteins (p72, p30, and p54) in the different neutralization mechanisms observed with sera from convalescent pigs were also studied. Anti-p72 and -p54 sera neutralized the virus only before attachment to different susceptible cells (Fig. 4A). However, serum against p30 neutralized the virus equally when antibodies were incubated with the virus before or after attachment to cells (Fig. 4A). Experiments testing inhibition of attachment of ^{35}S-labeled virus by antibodies against these three proteins revealed that sera to p72 and p54 were able to inhibit about 60% of virus attachment in both Vero cells and pig macrophages (Fig. 4B). In contrast, the anti-p30 serum did not inhibit virus attachment to cells, as found with a negative control serum (Fig. 4B).

Finally, inhibition of virus internalization tested in proteinase K detachment experiments showed that more than 90% of the virus incubated postattachment with anti-p72 or -p54 was internalized into the cells after 4 h of incubation at 37°C (Fig. 4C). However, anti-p30 antibodies inhibited virus internalization more than 95% in both Vero cells and pig macrophages (Fig. 4C). Therefore, we may conclude that antibodies against p72 and p54 are able to inhibit a first step of the virus replication cycle related to virus attachment, while anti-p30 antibodies inhibit a second step related to virus internalization. These results correlate with the two neutralization mechanisms observed with convalescent swine sera and allows us to assign specific proteins to the different neutralization mechanisms.

It is assumed frequently that viruses whose attachment to cells is inhibited by antibodies do not show postattachment neutralization. However, some examples in which the two mechanisms are efficient in neutralization of the viral particles have been described (13). Many possible explanations may underlie the postattachment neutralization of ASFV. However, one of the most interesting possibilities is that new epitopes, which are relevant to neutralization, are exposed or formed de novo after a virus has bound to its receptor as described for Sindbis virus (15). Another possible explanation is the existence of a second virus receptor implicated in virus internalization but not in attachment. Antibodies interacting with the protein associated with the virus internalization-mediated binding receptor could neutralize the virus with the same efficiency as antibodies that block virus attachment. Experiments to analyze the possible role in the interaction of the ASFV proteins involved in neutralization with different cell receptors are in progress.

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