

## Neutralization Susceptibility of African Swine Fever Virus Is Dependent on the Phospholipid Composition of Viral Particles

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In this study we have investigated the generation of African swine fever (ASF) virus variants resistant to neutralizing antibodies after cell culture propagation. All highly passaged ASF viruses analyzed were resistant to neutralization by antisera from convalescent pigs or antibodies generated against individual viral proteins which neutralized low-passage viruses. A molecular analysis of neutralizable and nonneutralizable virus isolates by sequencing of the genes encoding for neutralizing proteins revealed that the absence of neutralization of high-passage viruses is not due to antigenic variability of critical epitopes. A comparative analysis of phospholipid composition of viral membranes between low- and high-passage viruses revealed differences in the relative amount of phosphatidylinositol in these two groups of viruses, independent of the cells in which the viruses were grown. Further purification of low- and high-passage viruses by Percoll sedimentation showed differences in the phospholipid composition identical to those found with the partially purified viruses and confirmed the susceptibility of these viruses to neutralization. The incorporation of phosphatidylinositol into membranes of high-passage viruses rendered a similar neutralization susceptibility to low-passage viruses, in which this is a major phospholipid. In contrast, other phospholipids did not interfere with high-passage virus neutralization, suggesting that phosphatidylinositol is essential for a correct epitope presentation to neutralizing antibodies. Additionally, the removal of phosphatidylinositol from a low-passage virus by a specific lipase transformed this virus from neutralizable to nonneutralizable. These data constitute clear evidence of the importance of the lipid composition of the viral membranes for the protein recognition by antibodies and may account in part for the past difficulties in reproducibly demonstrating ASF virus-neutralizing antibodies by using high-passage viruses. © 1997 Academic Press

### INTRODUCTION

African swine fever (ASF) virus 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 molecule shares several structural features with the DNA of poxviruses (Yáñez *et al.*, 1995). ASF virus induces approximately 100 polypeptides in pig macrophages, the natural host cells (Alcaraz *et al.*, 1992) and about 40 of these polypeptides have been described as being incorporated into the viral particle (Carrascosa *et al.*, 1985; Esteves *et al.*, 1986).

Until the present, it has been accepted that nearly all viruses have neutralization sites and only ASF virus and Marburg and Ebola viruses lack the ability to be neutralized by virus-specific antibodies (Dimmock, 1993). Little work has been done in ASF virus neutralization, presumably because early experiments were phrased and interpreted within the hypothesis that neutralizing antibodies are not induced by this virus (Hess, 1981; Viñuela, 1985).

However, other authors have demonstrated that different isolates of ASF virus are neutralized by convalescent swine immune serum and monoclonal antibodies (Ruiz-Gonzalvo *et al.*, 1986a,b; Zsak *et al.*, 1993; Gómez-Puertas *et al.*, 1996). Authors that showed neutralization described a persistent fraction of nonneutralized virus (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 some times heterologous viruses in pig macrophages (Ruiz-Gonzalvo *et al.*, 1986b). Interestingly, high-passage viruses were found to be resistant to neutralization by antibodies (Zsak *et al.*, 1993). The ASF virus proteins p72, p30, and p54 have been characterized as involved in the virus neutralization (Zsak *et al.*, 1993; Gómez-Puertas *et al.*, 1996), mediating at least two different neutralization mechanisms related with the inhibition of the attachment or the internalization of the virus (Gómez-Puertas *et al.*, 1996).

The relevance of neutralizing antibodies in protection is poorly understood. Previous scientific reports concerning the role of antibodies in ASF virus protection showed that when those antibodies are transferred through colostrum they conferred a degree of protection to the suck-

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ling piglets against virus challenge (Schlafer *et al.*, 1984a,b). Passive transfer experiments using anti-ASF virus immunoglobulin indicate that ASF virus antibodies alone protect pigs against lethal infection with the virulent E75 isolate (Onisk *et al.*, 1994). These experiments also suggest that this antibody-mediated protective effect involves a critical early event that delays disease onset.

Because of the past difficulties in reproducibly demonstrating ASF virus-neutralizing antibodies by different authors, we have investigated the differences in neutralization of virus isolates highly passaged in cell cultures which are preferentially selected for *in vitro* studies. Virus propagation in cell lines has been shown to modify important phenotypic properties of the virus such as replication capability in pig macrophages, the natural host cell (Alcaraz *et al.*, 1992). Our results indicate that neutralizing antibodies are able to neutralize low-passage but not high-passage viruses. This difference in neutralization susceptibility between low- and high-passage viruses is not due to antigenic variability of critical epitopes, and seems to be related to the phospholipid composition of virions which is different depending on the number of cell culture passages.

## MATERIALS AND METHODS

### Cells

Vero and monkey stable (MS) cells were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS). Porcine alveolar macrophages were collected by alveolar lavage (Carrascosa *et al.*, 1982) and kept frozen in liquid nitrogen until use. Macrophages were cultured 24 hr prior to inoculation in RPMI 1640 medium supplemented with 20% heat-inactivated swine serum and 10% FBS.

### African swine fever virus isolates

Various Spanish isolates of ASF virus (608, 1207, E75, E70, and 646) were adapted to growth in Vero or MS cell cultures. Viruses were classified in two separate groups, depending on the low or high number of passages in cell cultures. The low-passage viruses used were 608VR13, 1207VR15, 646VR20, and E70MS14 (13, 15, and 20 passages in Vero or 14 passages in MS cell cultures, respectively). The high-passage viruses used were 608VR79, E75VR86, 646VR85, and E70MS81 (79, 86, and 85 passages in Vero or 81 passages in MS cell cultures, respectively). The strain BA71V, passaged more than 100 times in Vero cell cultures and included in the high-passage viruses group, was obtained from E. Viñuela (CBMSO-CSIC, Spain). Recombinant viruses expressing  $\beta$ -galactosidase or  $\beta$ -glucuronidase chromogenic marker genes in the thymidine kinase locus of the viral genome (608VR $\Delta$ TK $\beta$ -gus, 1207VR $\Delta$ TK $\beta$ -gal, and Ba71V $\Delta$ TK $\beta$ -

gus) were obtained as described previously (Gómez-Puertas *et al.*, 1995) from the 608VR13, 1207VR15, and BA71V isolates, respectively.

### Swine immune sera

Four-month-old pigs were inoculated intramuscularly with  $10^6$  PFU of the 1207VR15 (two pigs) or  $10^3$  of the 608VR13 (two pigs) virus isolates or by the intranasal/oral route with  $10^{5.6}$  50% tissue culture infectious doses (TCID<sub>50</sub>) of the E75CV1-4 isolate (four pigs). Sera from the pigs were collected 20 days postinoculation, heat inactivated (56°, 30 min), and stored at -20°.

Sera against recombinant proteins p30 and p54 expressed in baculovirus and against protein p72 expressed in *Escherichia coli* were obtained from pigs immunized as described (Gómez-Puertas *et al.*, 1996).

### DNA sequencing

Amplification of p72, p30, and p54 genes from different virus isolates was carried out by PCR with specific primers including the recognition sequences for the *Eco*RI and *Bam*HI restriction enzymes used for cloning the genes in pUC19 according to standard procedures (Sambrook *et al.*, 1989). The DNA templates used for all amplifications were obtained from supernatants of infected cell cultures. The cloned genes were sequenced by the dideoxynucleotide chain terminator method with M13-specific primers and internal p72, p30, or p54 gene primers.

### Neutralization assay

The neutralization activity of the sera was measured either in Vero cell monolayers or in pig macrophage cell cultures essentially as described previously (Gómez-Puertas *et al.*, 1995). The frozen stock of the different virus isolates was thawed and sonicated to avoid formation of viral aggregates. Then, 100 PFU of the virus were mixed with heat-inactivated immune swine sera at different dilutions for 18 hr at 37° and inoculated onto the cell cultures. The mixtures were incubated for 2 hr at 37° and then the Vero cells were covered with 0.8% agarose in DMEM 5% FBS. Pig macrophage cultures inoculated with the recombinant viruses by same procedure were supplemented with RPMI 1640 in presence of 20% heat-inactivated swine serum and 10% FBS. Number of plaques formed on Vero cell monolayers, as well as X-gal or X-gluc blue-stained individual macrophages, was counted, comparing them with those that appeared when the viruses were mixed with heat-inactivated preimmune swine serum used as control. The resulting percentages of neutralization are expressed as the following formula:

% neutralization = 100

$$= \frac{100 \times \text{number of plaques or blue cells in presence of immune serum}}{\text{number of plaques or blue cells in presence of preimmune serum}}$$

### Analysis of phospholipid composition of virions

Vero cells ( $10^7$ ) as well as alveolar pig macrophages ( $5 \times 10^7$ ) were infected with  $2 \times 10^6$  PFU of different viruses. After 2 hr, inoculum was washed and 20 ml of fresh culture medium containing 0.5 mCi of  $^{32}\text{PO}_4$  (Amersham, UK) was added. Uninfected Vero cells and pig macrophages were also labeled with  $^{32}\text{PO}_4$  and used as control. At 24 hr postinfection, extracellular virions were sedimented through a sucrose cushion of 20% (w/v). Lipids were chloroform-extracted and analyzed by one- and two-dimensional thin-layer chromatography (TLC) (Bernard *et al.*, 1984), using the following running buffers. One-dimensional TLC, chloroform:methanol:water 65:25:4; two-dimensional TLC, chloroform:methanol:water 65:25:4 (first dimension) and butanol:acetic acid:water 60:20:20 (second dimension). The spots were localized both by autoradiography and by colocalization with unlabeled carrier lipids after iodine staining.

Highly purified viruses were obtained by Percoll sedimentation as described previously (Carrascosa *et al.*, 1985) from extracellular  $^{32}\text{P}$ -labeled virus, and their phospholipid composition was analyzed as above. The membrane phospholipid composition of extracellular  $^{32}\text{P}$ -labeled vesicles released to the extracellular medium from infected cells, and purified also by Percoll, was also analyzed by the same procedure.

### Modification of lipid composition of virions

Ten micrograms of chloroform-diluted L- $\alpha$ -phosphatidylcholine (dipalmitoyl), L- $\alpha$ -phosphatidyl-L-serine, L- $\alpha$ -phosphatidylethanolamine, or L- $\alpha$ -phosphatidylinositol (all from Sigma Chemicals) was  $\text{N}_2$  dried and emulsified in 50  $\mu\text{l}$  of PBS by sonication. To incorporate the emulsified phospholipids into the viral particles, one volume of lipids was mixed with an equal volume of virus containing approximately 100 PFU and sonicated briefly. The incorporation of lipids into the viral particles was confirmed using [ $^{14}\text{C}$ ]phosphatidylinositol (NEN) as radioactive tracer and subsequent analysis of viral membrane composition by thin-layer chromatography and radioactive counting. After the lipid incorporation, viruses were sedimented through a sucrose cushion of 20% (w/v), incubated with antisera, and assayed in a neutralization assay.

Phosphatidylinositol from an extracellular low-passage virus purified by Percoll sedimentation was removed from the viral membrane by treatment with 5 units of phospholipase C, phosphatidylinositol-specific (PI-PLC; Sigma), for 1 hr at 37° in PBS, pH 7.4. The virus after treatment was washed, titrated, and analyzed for phospholipid composition and neutralization susceptibility.

## RESULTS

### High-passage viruses are not neutralized by antisera

The five highly virulent wild-type ASF viruses 646, E70, E75, 608, and 1207 were adapted to growth in Vero or

MS cells and serial passages of the viruses were carried out. Virus populations after low (less than 25 passages) and high (more than 75 passages) numbers of passages were analyzed comparatively in a neutralization assay to characterize differences in neutralization susceptibility of the viruses. Additionally, the virus isolate BA71V, passaged more than 100 times in Vero cells, was analyzed. For the comparative neutralizations we used eight hyperimmune sera generated by inoculation of pigs with attenuated viruses, as well as antisera generated in pigs by immunization with the recombinant proteins p72, p30, or p54 (two sera each).

The serum neutralization results in Vero cells, quantified by the percentage of plaque reduction produced by the antisera diluted 1:5, are shown in Fig. 1. Interestingly, high-passage viruses exhibited extremely low levels of neutralization (less than 20% of their infectivity) with the same sera that were able to neutralize the low-passage viruses. Either polyclonal or monospecific antisera showed similar quantitative differences between percentages of neutralization of low- and high-passage viruses.

To analyze whether or not the cell type influenced the differences in the percentages of neutralization detected between low- and high-passage viruses, we compared the percentages of neutralization obtained in Vero cells with those showed in pig macrophages, the natural host cells for ASF virus. For these experiments with pig macrophages we used a modified neutralization assay for these primary cultures in which recombinant ASF viruses expressing chromogenic marker genes are used (Gómez-Puertas *et al.*, 1995). The low-passage 608VR $\Delta\text{TK}\beta$ -gus and 1207VR $\Delta\text{TK}\beta$ -gal and the high-passage Ba71V $\Delta\text{TK}\beta$ -gus recombinant viruses were used; the percentage of neutralized virus particles was calculated by detection of infected cells by chromogenic dye staining at 14 hr postinfection. The phenomenon of absence of neutralization of high-passage viruses was also reproduced in pig macrophages, similar to what was observed in Vero cells (Fig. 2).

A possible explanation for the absence of neutralization found in high-passage viruses could be the antigenic variability of critical epitopes in neutralization during cell culture propagation. To analyze this hypothesis, we sequenced the p72, p30, and p54 genes from neutralizable and nonneutralizable viruses (labeled with an arrow in Fig. 1). Sequence analysis of these genes from viruses belonging to both groups of neutralizable and nonneutralizable viruses showed a 100% homology (not shown).

### Cell culture propagation modifies the phospholipid composition of the ASF virus

A second hypothesis for explaining the lack of neutralization activity of antisera in high-passage viruses could be the masking of or alterations in the presentation of

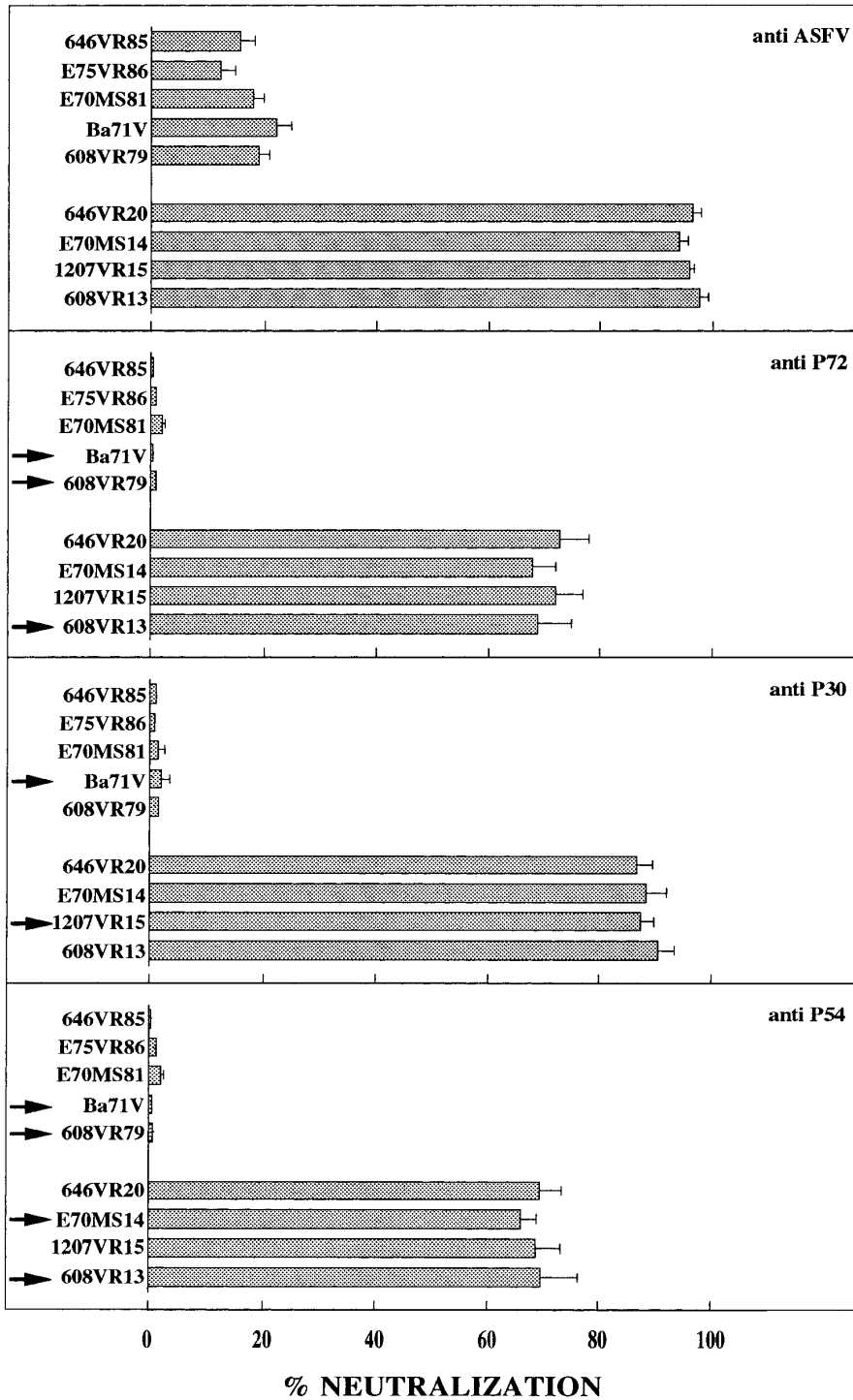


FIG. 1. Comparison of the neutralization percentages of different high- and low-passage isolates obtained with neutralizing antisera in Vero cells. Five high-passage (646VR85, E75VR86, E70MS81, BA71V, and 608VR79) and four low-passage (646VR20, E70MS14, 1207VR15, and 608VR13) virus isolates were subjected to a neutralization assay with eight different polyclonal sera from infected animals (see Materials and Methods) as well as with monospecific antisera raised to proteins p72, p30, and p54 (two each), all of them diluted 1:5. The neutralization percentage was determined using a plaque reduction assay over cultured Vero cells. The means  $\pm$  SE of the values obtained using the eight polyclonal (anti-ASF virus) or the monospecific (anti-p72, anti-p30, anti-p54) sera in three independent experiments are shown. Arrows indicate ASF virus strains whose respective genes coding for p72, p30, or p54 proteins were sequenced.

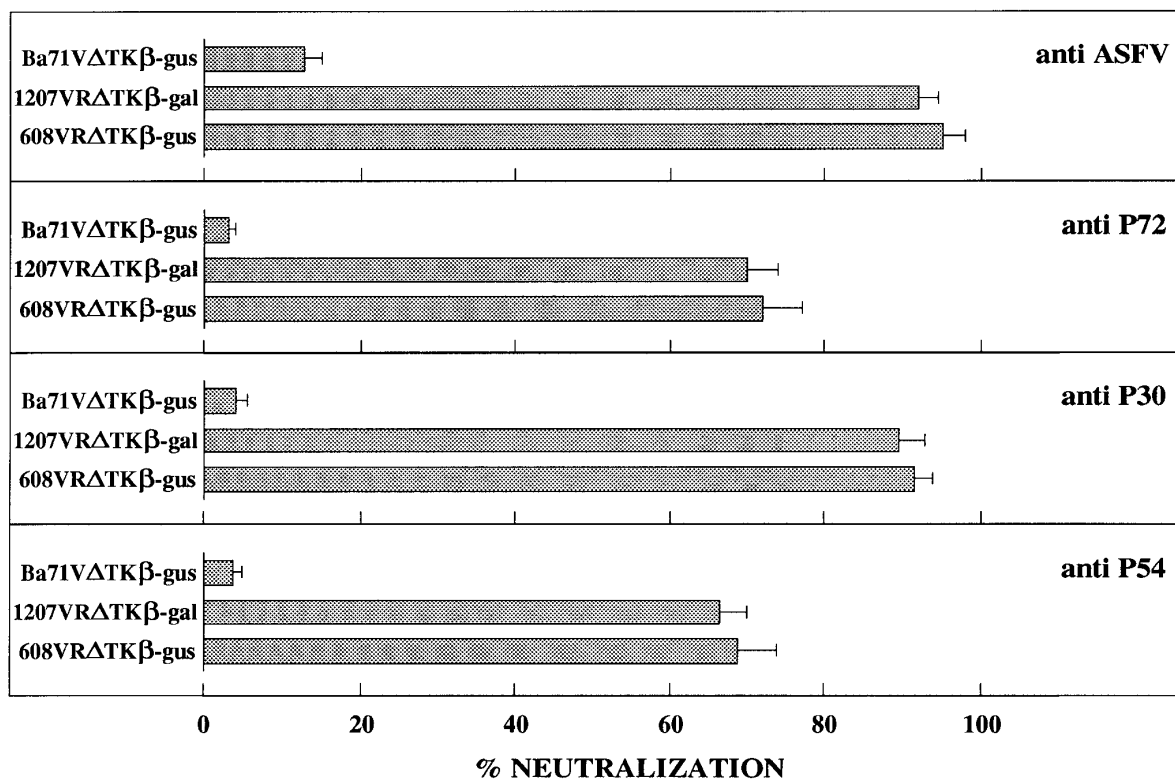


FIG. 2. Comparison of the neutralization percentages of three recombinant high- and low-passage virus isolates obtained with neutralizing antisera in alveolar macrophages. One high-passage (Ba71VΔTKβ-gus) and two low-passage (1207VRΔTKβ-gal and 608VRΔTKβ-gus) recombinant viruses, expressing the marker genes β-gus or β-gal, were assayed for neutralization with the same polyclonal and monospecific sera as in Fig. 1. The neutralization percentage was determined using a color-based assay determining individual infected pig macrophages. The means ± SE of the values obtained with the sera in three independent experiments are shown.

antigenic determinants by lipids (Stolze *et al.*, 1987). To explore that, we compared the phospholipid composition of neutralizable (low-passage) and nonneutralizable (high-passage) virions. ASF virions labeled with  $^{32}\text{P}$  were sedimented from infected cell culture supernatants and lipids were extracted and analyzed by thin-layer chromatography and compared with the phospholipid pattern obtained from plasma membranes of Vero cells and alveolar pig macrophages. Three major phospholipids were identified in the preparations of the low (608VR13, 646VR20)- and high-passage (608VR79, 646VR85, Ba71V) viruses (Fig. 3A). The two major  $^{32}\text{P}$ -labeled phospholipids detected in membranes of both Vero cells and macrophages were identified by comigration with phosphatidylcholine and phosphatidylethanolamine markers. Phosphatidylcholine was the major phospholipid component of low- and high-passage virus membranes, phosphatidylethanolamine being a less abundant phospholipid in the virions. However, a clear difference between low- and high-passage virus phospholipid composition was a third phospholipid not detectable in cell plasma membranes that was major in the low-passage viruses but present only in small amounts in the nonneutralizable high-passage viruses. This phospholipid was identified by two-dimensional thin-layer chromatography using

specific markers as phosphatidylinositol (Fig. 3B). This phospholipid constitutes about 40% of the total lipid composition of low-passage virus membranes, while makes up only 10% of the high-passage virions as deduced from densitogram of radioactive spots obtained from labeled samples (Fig. 3C).

To study if differences in the phospholipid composition found between viruses could be due to contaminants in the virus preparations, we purified by Percoll sedimentation the viruses 608VR13 and Ba71V, labeled with  $^{32}\text{P}$  (Figs. 4A and 4B). The viral membranes of these highly purified viruses contained similar phospholipid composition found in the more crude virus preparations, confirming the previous data about differences between low- and high-passage viruses (Fig. 4C). The analysis of phospholipid composition of the membranes of extracellular vesicles released from infected cells and purified by Percoll gradients (Carrascosa *et al.*, 1985) revealed that phosphatidylinositol was absent in these vesicles which usually contaminate the virus preparations (Fig. 4C). Interestingly, infection with the high-passage virus released to the extracellular medium about five-fold more vesicles than the low-passage virus (Figs. 4A and 4B). The highly purified viruses maintained the same neutralization susceptibility pattern observed before purification (Fig. 4D).

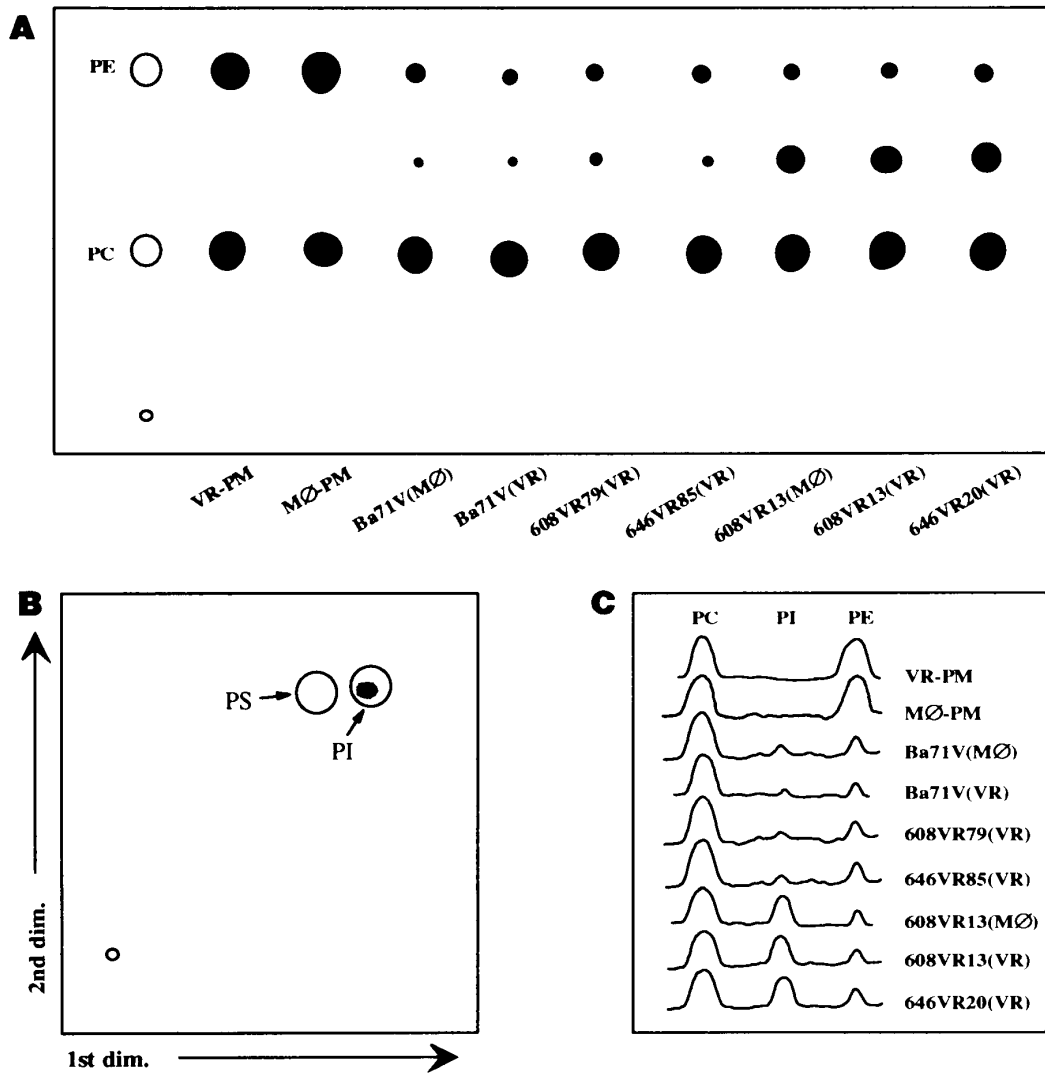


FIG. 3. Comparative analysis of phospholipid composition of low- and high-passaged virions. (A) Autoradiogram of one-dimensional thin-layer chromatography of  $^{32}\text{P}$ -phospholipids extracted from Vero cell plasma membrane (VR-PM); 608VR13, 608VR79, 646VR20, 646VR85, and Ba71V extracellular virions grown in Vero cell culture [608VR13(VR), 608VR79(VR), 646VR20(VR), 646VR85(VR), and Ba71V(VR), respectively]; alveolar macrophage plasma membrane (MØ-PM); and 608VR13 and Ba71V extracellular virions grown in alveolar macrophage cultures [608VR13(MØ) and Ba71V(MØ), respectively]. Positions of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) markers, as well as origin (O), are indicated. (B) Autoradiogram of two-dimensional thin-layer chromatography of the pooled intermediate spots obtained in A. Positions of phosphatidylserine (PS) and phosphatidylinositol (PI) markers, as well as origin (O), are indicated. (C) Densitometric tracings of the autoradiogram showed in A. PC, PI, and PE peaks are indicated.

### Modifications of the phospholipid composition of virions affect their neutralization susceptibility

The role of phospholipids in the inhibition of ASF virus neutralization by masking viral epitopes was examined by experiments of incorporation of different phospholipids into purified ASF virus. Phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, or phosphatidylinositol were incorporated before neutralization into seven ASF viruses, two of them belonging to the low-passaged neutralizable group and five to the high-passaged nonneutralizable group. To assess that phospholip-

ids were incorporated into the viral particles, we analyzed viral membranes after incubation with emulsified  $^{14}\text{C}$ -labeled phosphatidylinositol. This phospholipid was efficiently incorporated into the viral particle membranes (data not shown).

As a result of these experiments, we showed that phosphatidylinositol incorporation into the viral particle rendered the high-passaged virus neutralizable at levels similar to those shown with the neutralizable low-passaged viruses (Fig. 5). However, incorporation of the other phospholipids to the viral particles did not affect the neutralization susceptibility of the low-passaged viruses (Fig.

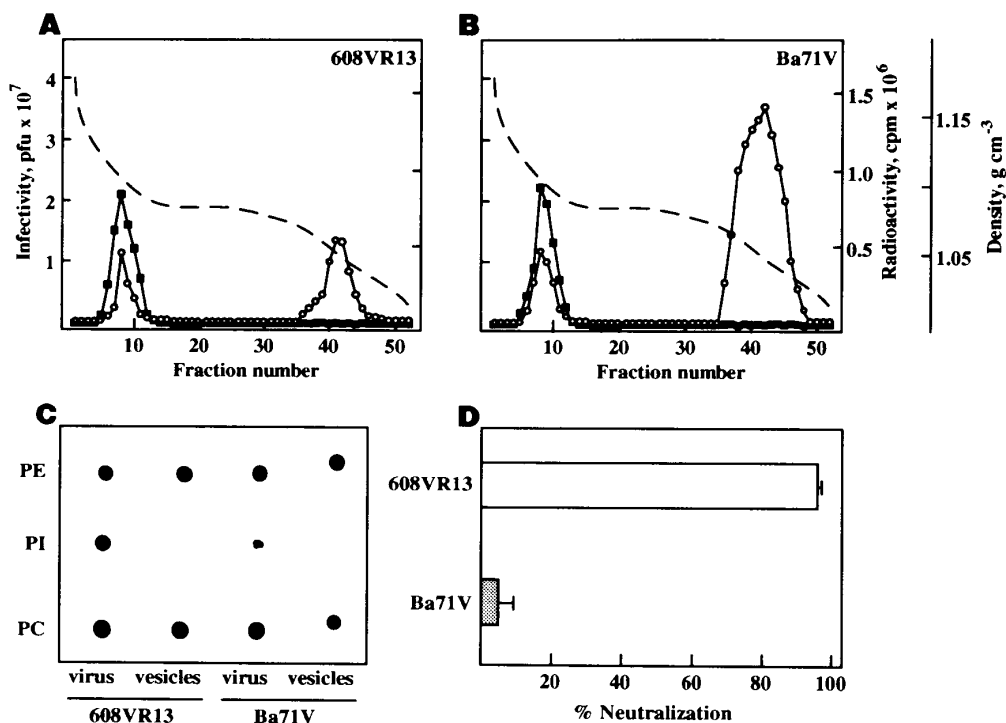


FIG. 4. Purification of extracellular  $^{32}\text{P}$ -labeled ASF virus by Percoll sedimentation. Extracellular  $^{32}\text{P}$ -labeled 608VR13 (A) or Ba71V (B) ASF viruses obtained from one roller each of Vero cells were subjected to Percoll density gradient, and 200- $\mu\text{l}$  fractions were collected from the bottom. Portions of each fraction were taken to determine density (dashed line), infectious virus titers (solid squares), and total radioactivity (open circles). (C) Autoradiogram of one-dimensional thin-layer chromatography of  $^{32}\text{P}$ -phospholipids extracted from fractions containing the virus ( $\rho \approx 1.095$  g/ml) and secreted vesicles ( $\rho \approx 1.04$  g/ml) of the Percoll density gradients showed in A and B. (D) Comparison of the neutralization percentages of the Percoll-purified 608VR13 and Ba71V viruses obtained with neutralizing antisera at 1:5 dilution on Vero cells monolayer. The means  $\pm$  SE of the values obtained with the sera in three independent experiments are shown.

5). Infectivity of the virus after incorporation of the different phospholipids was not affected in absence of immune sera (not shown).

The above results demonstrate that enrichment of high-passage viruses with phosphatidylinositol may recover the susceptibility of these viruses to neutralization by antibodies while other phospholipids seem not to interfere with the epitope presentation to antibodies.

To study the effect of phosphatidylinositol removal on a neutralizable virus, we incubated a Percoll-purified low-passage virus (608VR13) with PI-PLC, which removes specifically phosphatidylinositol. A phospholipid analysis of the resulting viral membranes after the treatment revealed a complete removal of phosphatidylinositol from the virions (Fig. 6A). Interestingly, a comparative analysis of the neutralization susceptibility of this purified virus before and after lipase treatment showed that the removal of phosphatidylinositol transformed this virus from neutralizable to nonneutralizable (Fig. 6B).

## DISCUSSION

We have shown in this study that cell culture propagation of ASF virus generates virus variants that are resistant to neutralization by antibodies which otherwise are

capable of neutralizing low-passage viruses. Until now it has been generally assumed that ASF virus-infected pigs do not produce neutralizing antibodies (Dimmock, 1993). Our observation that the absence of neutralization is a specific phenomenon of high-passage viruses may in part account for past difficulties in reproducibly demonstrating ASF virus-neutralizing antibodies (Hess, 1981; Viñuela, 1985). High-passage viruses, such as BA71V, one of the most studied ASF virus isolates, which produce plaques in less than a week (Gómez-Puertas *et al.*, 1995), are preferentially selected for *in vitro* studies. In contrast, low-passage viruses present difficulties in plaque formation in tissue cultures (Gómez-Puertas *et al.*, 1995), and they are not generally selected for *in vitro* neutralization assays. These differential neutralization patterns between high- and low-passage ASF viruses suggest caution in the choice of the virus isolate to be used in the neutralization studies. Additionally, in other viral models, some authors assume the neutralization differences depending on whether or not antibodies interfered with the ability of the virus to infect different cells (Grady and Kinch, 1985). We have not detected differences in neutralization of low- and high-passage ASF virus strains depending on host cells.

Other examples of neutralization dependence on pas-

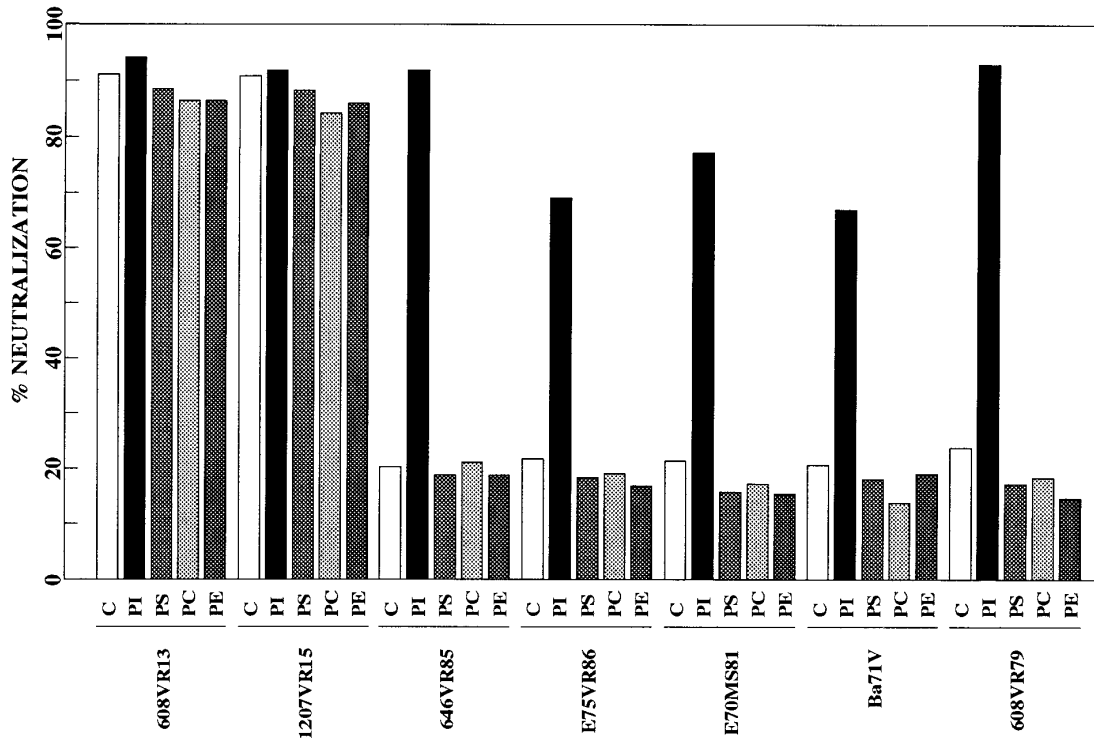


FIG. 5. Neutralization susceptibility of phospholipid-modified virions. 100 PFU of two low-passage (1207VR15 and 608VR13) and five high-passage (646VR85, E75VR86, E70MS81, BA71V, and 608VR79) virus isolates was mixed with 10  $\mu$ g of emulsified L- $\alpha$ -phosphatidylinositol (PI), L- $\alpha$ -phosphatidyl-L-serine (PS), L- $\alpha$ -phosphatidylcholine (PC), or L- $\alpha$ -phosphatidylethanolamine (PE), as described under Materials and Methods. The viruses were sonicated, ultracentrifugation semipurified, and subjected to neutralization on Vero cell monolayers in presence of a pool of the eight polyclonal anti-ASF virus sera used in this study, diluted 1:5. The neutralization percentages of the seven strains analyzed after modification with the different phospholipids (PI, PS, PC, and PE) or without modification (C) are shown.

sage history of the viruses and/or on the host system have been described (Baldinotti *et al.*, 1994; Grady and Kinch, 1985; Kim *et al.*, 1994). For instance, recently, this phenomenon has been described in the feline immunodeficiency virus (Baldinotti *et al.*, 1994), although the results presented by these authors are the opposite from that found in our virus model. Sera from feline immunode-

ficiency virus-infected cats efficiently neutralized high-passage viruses but not low-passage viruses. Interestingly, after replication in cats, the high-passage virus showed behavior in neutralization identical to that of the low-passage virus.

Generally, the influence of target cells on neutralization has been attributed to the fact that the virus uses different

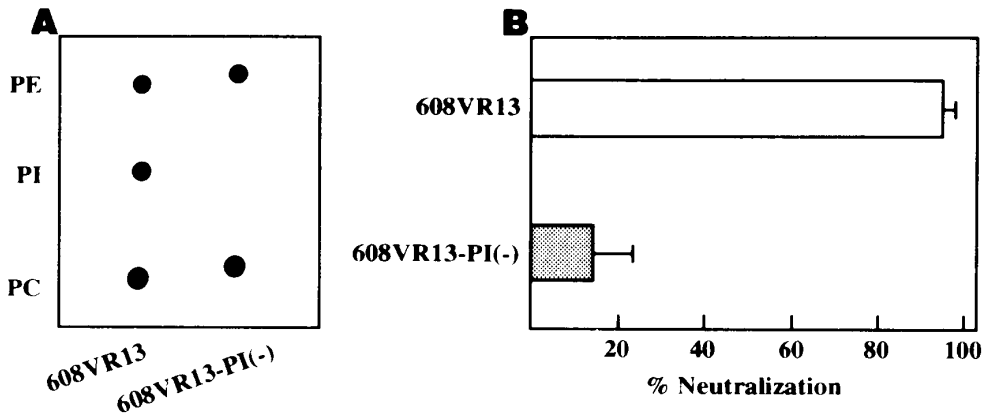


FIG. 6. Effect of the PI-PLC treatment on neutralization susceptibility. (A) Autoradiogram of one-dimensional thin-layer chromatography of  $^{32}$ P-phospholipids extracted from Percoll-purified 608VR13 virus after (608VR13) and before [608VR13-PI(-)] the PI-PLC treatment. (B) Comparison of the neutralization percentages of the same viruses as in A obtained with neutralizing antisera at 1:5 dilution on Vero cells monolayer. The means  $\pm$  SE of the values obtained with the sera in three independent experiments are shown.



receptors to infect the cells (Dimmock, 1993). In this case we cannot rule out that ASF virus during cell culture propagation acquires alternative ways of entering the susceptible cells. A possible mechanism to create a new viral attachment protein could be the change in location of a structural protein. This feature could make the neutralizing antibodies generated during infection inefficient against other proteins with similar function in wild-type but not in adapted viruses. We cannot rule out that modifications in the lipid composition of virions could modify the topology of viral particles making critical antigens in low-passage viruses relevant again.

As has been described in other viral systems (Luan *et al.*, 1995), ASF virus seems to direct the phospholipid composition of its membrane, since low- and high-passage viruses growing for several passages in the same cells contain different relative percentages of phospholipids in their membranes. The analysis of phospholipid composition of high- and low-passage virus membranes, grown in the same cells, showed clear differences in the relative percentages of the phospholipids. While low-passage viruses contain a high percentage of phosphatidylinositol in their membrane, this phospholipid is only a minor component of the membranes of high-passage nonneutralizable viruses.

In Aleutian disease virus, lipid composition of virions conditioned their capability to be neutralized (Stolze *et al.*, 1987). Reconstitution of detergent-extracted virus with different lipids either did or did not inhibit its neutralization by antisera. In that case, phosphatidylserine and phosphatidylcholine inhibited the virus neutralization in a dose-dependent manner. This phenomenon could be similar to that observed in ASF virus, in which certain phospholipids could interfere with the virus neutralization. In addition, the presence of high amounts of phosphatidylinositol (PI) in the low-passage virions suggests that ASF virus membranes are derived from the endoplasmic reticulum (ER) compartment of the host cell. This idea is supported by the phospholipid composition of the newly formed virions that is similar to the known membrane composition of the ER, where essentially all PI is synthesized (van Meer, 1989). However, the high-passage, nonneutralizable virions have in contrast a phospholipid composition that is intermediate between that of the ER membrane and that of later secretory compartments (van Meer, 1989). This finding suggests that a different route of membrane formation could be followed between low- and high-passage viruses (Sodeik *et al.*, 1993).

Phospholipids have been shown to be crucial for antigenic properties of hepatitis B surface antigen (HBsAg). All epitopes of this protein defined by a panel of monoclonal antibodies showed variation of reactivity after reconstitution with acidic phospholipids (Gómez-Gutiérrez *et al.*, 1994, 1995). Electrostatic interactions between HBsAg proteins and acidic phospholipids are partly re-

sponsible for the complete recovery of the antigenic activity. These authors suggest that the antigenic activity is dependent on the physical state of the phospholipid moiety. Once the conformation of the antigen in membranes is established, additional interactions imparted by the various phospholipids may give a difference in the pattern of antigenicity as shown with HBsAg (Gómez-Gutiérrez *et al.*, 1994, 1995) or with epitopes involved in neutralization of ASF virus.

After this study, it is strongly suggested that the apparent lack of neutralization of high-passage ASF viruses is due to masking of viral epitopes by the relative composition of different phospholipids in viral membranes. The conversion of nonneutralizable to neutralizable viruses by incorporation of phosphatidylinositol suggests that the concentration of this phospholipid in the virion is crucial for antigen presentation to antibodies. Therefore, the absence of this lipid or lower concentrations than those required for a correct epitope presentation might be responsible for the ASF virus escape from neutralization. The absence of apparent effects on neutralization susceptibility after incorporation of phosphatidylethanolamine, phosphatidylcholine, or phosphatidylserine on neutralizable low-passage naturally phosphatidylinositol-enriched ASF viruses suggests that the presence of phosphatidylinositol is necessary for a correct epitope presentation of the viral antigens and, once the adequate protein–phosphatidylinositol interaction is established, the presentation is not affected by the incorporation of other phospholipids. Furthermore, the removal of phosphatidylinositol from virions also had drastic effects on neutralization susceptibility.

Phosphorylated products of phosphatidylinositol have been shown to play critical roles in the regulation of membrane traffic and protein conformation (for review see De Camilli *et al.*, 1996). Although the affinities involved in the interactions between proteins and phosphatidylinositol are not thought to be very high (Ferguson *et al.*, 1995a,b), these interactions may cooperate with protein–protein interaction mechanisms, and therefore could account for spatial specificity.

Our findings suggest that lipids play an important role in the presentation of viral epitopes to the immune system and probably will have to be considered in the analysis of humoral immunity in different viral models in which cell-passaged virus isolates are currently used. In addition, the phenomenon of epitope masking by altering lipid composition of virus membranes could have *in vivo* relevance because it may constitute a novel mechanism used for viruses to evade the immune system.

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