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Blocking antibodies inhibit complete African swine fever virus neutralization

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Abstract

A persistent non-neutralized African swine fever virus (ASFV) fraction is found with most convalescent swine sera in in vitro neutralization assays. To study this phenomenon, antisera from convalescent pigs infected with different virus isolates and showing complete or incomplete virus neutralization were used. Different experiments determined that incomplete neutralization of ASFV is caused neither by virus aggregation, nor low affinity or stability of virus-antibody complexes. Additionally, attempts to purify antigenic escape mutant viruses from the persistent fraction was also unsuccessful. Nevertheless, competition experiments between sera demonstrated that antibodies present in sera showing persistent fraction inhibited the complete neutralization mediated by antibodies present in sera which neutralize 100% of virus infectivity. These results suggest that induction of blocking antibodies during ASFV infection could represent the main cause for the persistent surviving virus fraction observed in neutralization assays and could also explain the persistent infections observed in some convalescent pigs. © 1997 Elsevier Science B.V.

Keywords: African swine fever virus; Neutralization; Blocking antibodies

1. Introduction

African swine fever virus (ASFV), the causative agent of African swine fever, produces an economically significant disease of swine that is endemic in many African countries and others in the mediterranean area. It is an icosahedral cytoplasmic deoxivirus formed by a single molecule of DNA of about 170 kilobases (kb) which molecule shares several structural features with the DNA of poxviruses and contains about 150 open reading frames (Yáñez et al., 1995). The infection of domestic pigs with virulent ASFV strains leads to a highly lethal disease.

The relevance of neutralizing antibodies in protection is poorly understood. Previous scientific reports concerning the role of antibodies in ASFV

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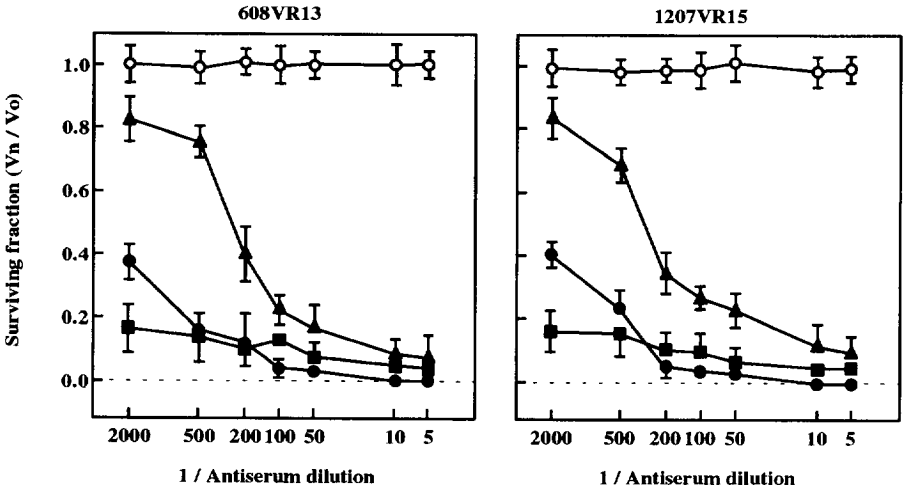

Fig. 1. Persistent surviving virus fraction after neutralization assays using different anti-ASFV sera. One hundred PFU of two ASFV strains (608VR13 and 1207VR15) were subjected to a neutralization assay using distinct dilutions of three different pools of polyclonal sera obtained from surviving animals to inoculation with three attenuated ASFV strains (s608, solid circles; s1207, solid squares; sE75, solid triangles) or from a non infected animal (control, open circles). The surviving fraction was determined using a plaque reduction assay over cultured Vero cells. The figure shows the mean ± S.E. of the values obtained in three independent experiments.

protection showed that when those antibodies are transferred through the colostrum a degree of protection to the suckling piglets against virus challenge is conferred (Schlafer et al., 1984a,b). Passive transfer experiments using anti-ASFV immunoglobulin indicate that specific antibodies alone protect pigs against lethal infection (Onisk et al., 1994). These experiments also suggest that this antibody-mediated protective effect involves a critical early event that delays disease onset. Sera from pigs inoculated with attenuated strains of ASFV inhibit the infection (Ruiz-Gonzalvo et al., 1986a) or neutralize the virus (Gómez-Puertas et al., 1995, 1996; Ruiz-Gonzalvo et al., 1986b; Zsak et al., 1993) in in vitro assays. Authors that studied neutralization described a persistent surviving fraction of non-neutralized virus of about 10%. The inability of this virus to be completely neutralized by antibodies could lead to a very frequently described chronic ASFV infection in pigs, in which infectious virus persists even in the presence of an excess of neutralizing antibodies (Carrillo et al., 1994).

2. Results and discussion

To study the generation of the surviving fraction in in vitro neutralization assays, we developed several neutralization experiments using three pools of different swine anti-ASFV sera and two cell-adapted ASFV strains, previously used to analyze the ASFV neutralization mechanisms (Gómez-Puertas et al., 1996). The three pools of sera were obtained from pigs inoculated intramuscularly with 10^6 PFU of the 1207VR15 (two pigs) or 608VR13 (two pigs) virus isolates or orally with $10^{5.6}$ 50% tissue culture infectious dose ($TCID_{50}$) of the E75CV1-4 isolate (four pigs). Sera from pigs inoculated with 1207VR15 (s1207) or 608VR13 (s608) were collected at 20 days post-inoculation. Sera from pigs inoculated with virus E75CV1-4 (sE75) were collected after no viremia was detected (about four weeks after infection). All sera were heat-inactivated (56°C, 30 min) and stored at −20°C until used. The pools were generated mixing equal quantities of each homologous serum. Serum from a pig before immunization was used as control preim-

mune serum. The ASFV strains 608 and 1207, adapted to growth on Vero cell cultures and used after a maximum of 20 passages (608VR13, 1207VR15) were used for the neutralization assays.

The neutralization assays were performed on Vero cell monolayers 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 immune swine sera at different dilutions for 18 h at 37°C, and then inoculated to the cell cultures. After 2 h of virus adsorption, Vero cells were washed and covered with 0.8% agarose in DMEM supplemented with 5% fetal bovine serum (FBS). The number of virus-induced plaques in the presence of antibodies (V_n) and the number of plaques induced in the absence of antibodies (V_0) were used to calculate the surviving fraction (V_n/V_0) (Massey and Schochetman, 1981).

The results of those neutralization experiments, which are summarized in Fig. 1, showed that a surviving persistent fraction of 0.0 was only obtained with s608 pool of sera (s608) that was able to neutralize totally the viruses 608VR13 and 1207VR15 at low serum dilutions. However, the other two analyzed pools of sera showed a persistent fraction of 0.05–0.2 at the same dilutions. Percentages of non-neutralized virus fractions obtained with the three pool of sera were representatives of the percentages found with the different sera individually tested (data not shown).

Table 1
Surviving fraction ($(V_n/V_0) \times 10^2$) after neutralization of different amounts of the 608VR13 or 12O7VR15 ASFV strains with the antisera sE75, s1207 or s608

ASFV strain	PFU/assay	Antisera at dilution 1:5		
		sE75	s1207	s608
608VR13	10^1	12.4 ± 5.8*	9.7 ± 2.5	0.0 ± 0.0
	10^2	13.1 ± 4.1	8.8 ± 3.3	0.0 ± 0.0
	10^3	12.9 ± 3.3	9.3 ± 3.5	0.0 ± 0.0
	10^4	13.5 ± 3.9	10.4 ± 3.4	0.0 ± 0.0
	10^5	13.3 ± 4.8	10.2 ± 4.8	1.2 ± 0.3
1207VR15	10^1	18.4 ± 0.7	10.5 ± 3.8	0.0 ± 0.0
	10^2	19.3 ± 5.5	10.4 ± 3.8	0.0 ± 00
	10^3	18.2 ± 4.5	12.1 ± 2.7	0.0 ± 0.0
	10^4	19.1 ± 2.2	12.0 ± 2.1	0.0 ± 0.0
	10^5	18.9 ± 1.5	11.8 ± 3.5	1.4 ± 0.5

* Data are represented as mean 1 ± S.E.M. of three independent experiments.

The existence of this persistent fraction in other viral models has been explained as caused by different factors such as: formation of viral aggregates non accessible to the antibodies (Taniguchi and Urasawa, 1987); incapability of the sera to neutralize more than a few virus particles (low titre antiserum; Narayan et al., 1984); generation of neutralization-resistant mutant variants of the virus (Hussain et al., 1987; Lambkin et al., 1994; Li et al., 1995; Watkins et al., 1993); low affinity of neutralizing antibodies, needing long time incubations to perform an effective neutralization (Torfason et al., 1992); low stability of the antibody-virus complex, reversing easily after dilution (Suñé et al., 1990); and existence of blocking antibodies that inhibit virus neutralization (Massey and Schochetman, 1981; O'Rourke et al., 1988). In this work, we have analyzed all those possibilities, trying to explain the surviving persistent fraction detected in ASFV neutralization assays.

First we investigated the existence of viral aggregates non accessible to antibodies. To eliminate this event, all neutralization experiments were performed with sonicated virus stocks to prevent virus clustering. For further elimination of virus aggregation, the virus stock, once sonicated, was filtered through a 0.4 μm filter. Due to the size of the ASFV particle, 0.2 μm (Carrascosa et al., 1984), viral aggregates of two or more virus particles are not able to pass through the filter. Then, comparative neutralization assays of 100 PFU of the 608VR13 ASFV strain before and after filtering were carried out with sE75 pool of sera. This experiment, performed in triplicate, showed that virus filtration did not affect the percentage of surviving virus fraction (before filtering: 0.09 ± 0.01; after filtering: 0.10 ± 0.01), suggesting that the existence of the ASFV persistent fraction is not due to viral aggregation. Similar results were obtained using s1207 pool of sera and 608VR13 and 1207VR15 virus strains (data not shown).

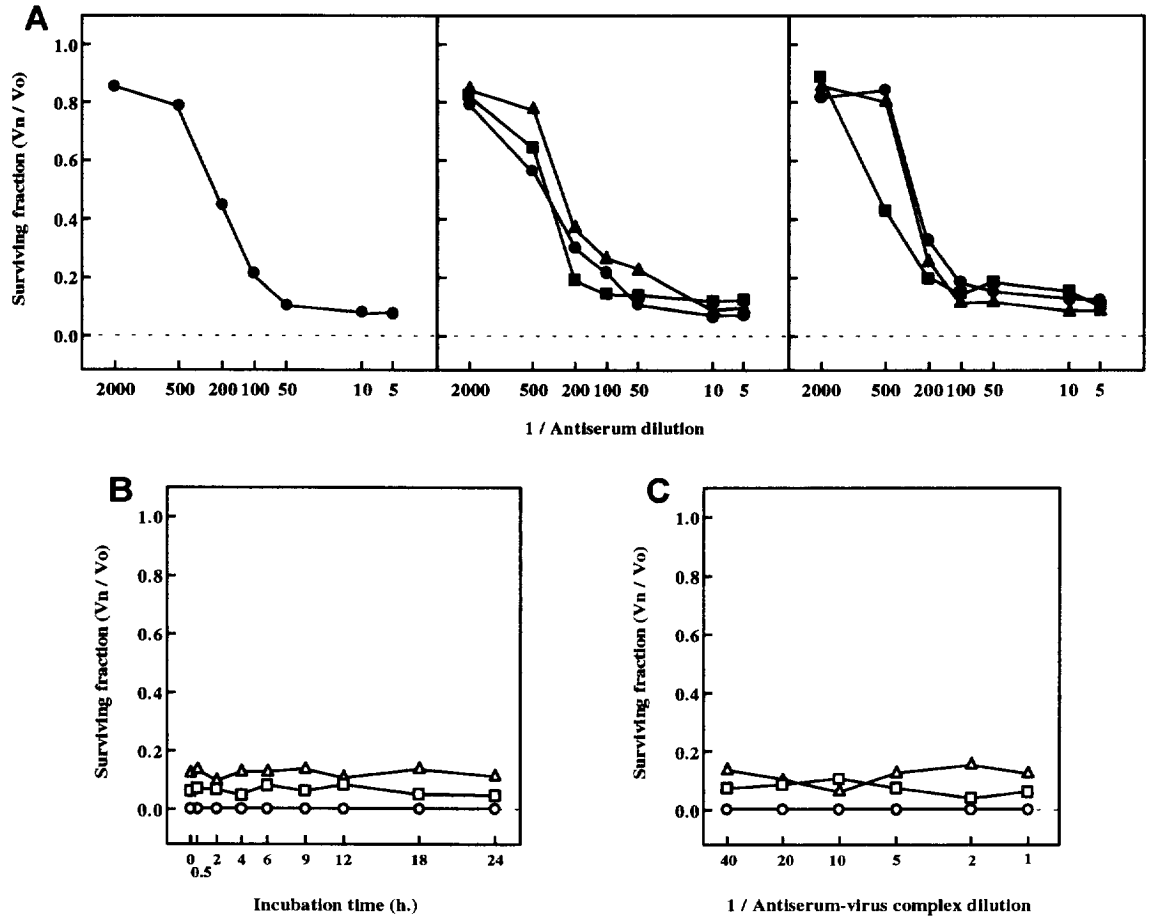

Fig. 2. (A) Three neutralization running of 1207VR15 virus isolate using the antiserum sE75. Left: surviving faction obtained after a neutralization assay of 100 PFU of the virus using different dilutions of the serum. Center: three neutralization resistant clones from the previous assay were again subjected to a neutralization experiment using the same serum. The resulting surviving virus fractions at different serum dilutions are represented. Right: three neutralization-resistant clones from the second neutralization running were submitted to a third neutralization experiment using the same serum. The figure represents the surviving fraction obtained in each dilution used. (B) Time dependence of virus-antibody complex formation in relation with the percentage of surviving fraction. One hundred PFU of 1207VR15 virus were mixed with the pools of sera sE75 (triangles), s1207 (squares) or s608 (circles) at 1:5 dilution and incubated during the times indicated in the figure. After that, the virus-antisera mixtures were layered on Vero cell cultures to analyze neutralization percentages. The figure shows the surviving virus fraction obtained in each case. (C) Effect of the virus-antibody complex dilution on the surviving fraction. One hundred PFU of 1207VR15 strain were mixed with the pools of sera sE75 (triangles), s1207 (squares) or s608 (circles) at 1:5 dilution, maintained at 37°C for 18 h, and diluted as indicated in the figure just before layering on Vero cell cultures. The figure shows the surviving fraction measured to each serum dilution.

To analyze the hypothesis of insufficient amount of neutralizing antibodies induction during infection, allowing incomplete neutralization of the virus, different quantities of 608VR13 or 1207VR15 virus strains were subjected to neutralization assays using the pools of antisera sE75, s1207 and s608 at 1:5 dilution. The results of this experiment are summarized in Table 1. As shown, the surviving fraction did not depend significantly of the number PFU of virus employed, suggesting that the quantity of neutralizing antibodies present in the different sera is not relevant for the persistent non-neutralized fraction.

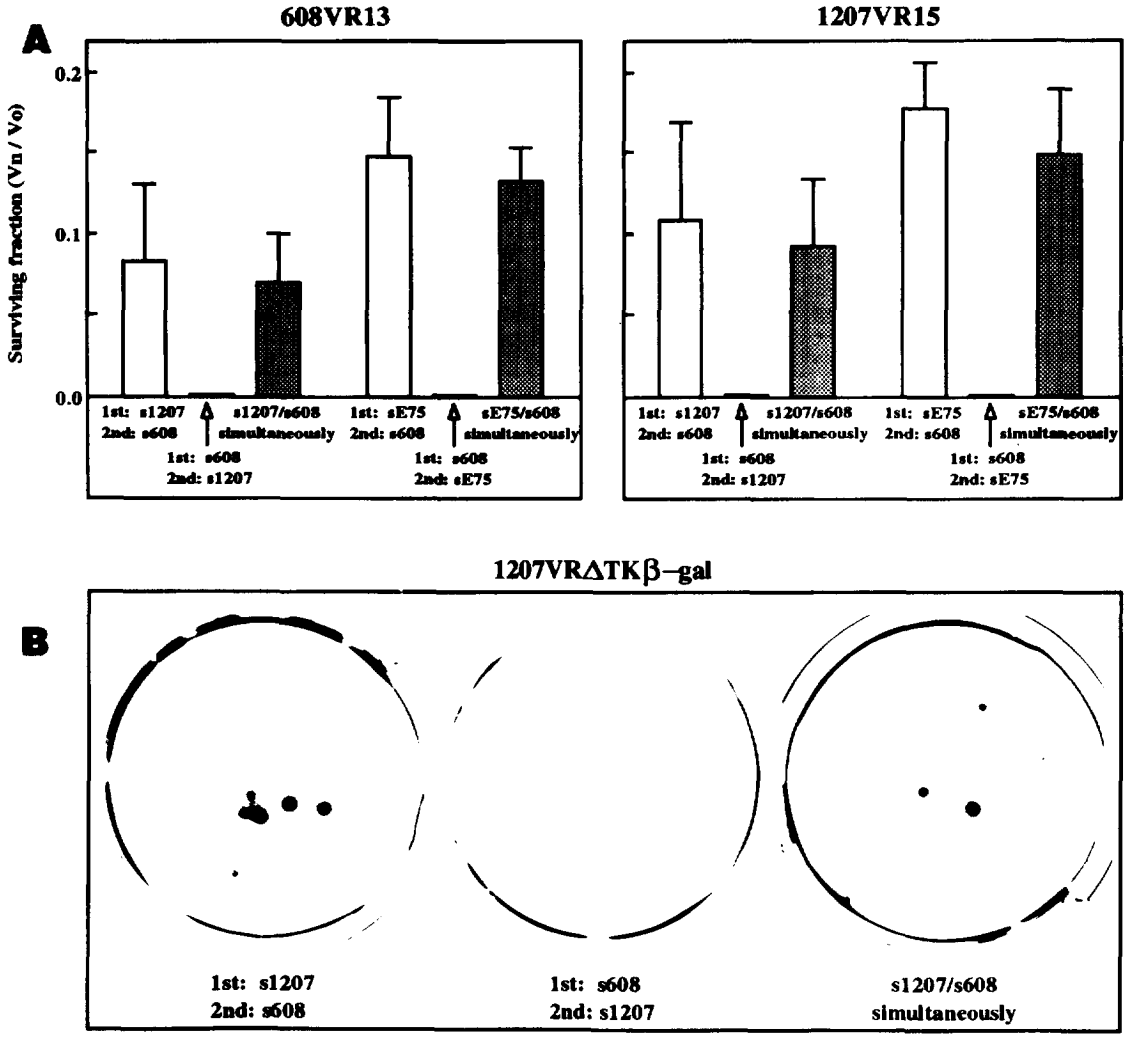

Fig. 3. Competition neutralization experiments between sera presenting different percentages of non-neutralized persistent virus fractions. (A) One hundred PFU of the ASFV isolates 608VR13 (left) or 1207VR15 (right) were subjected to neutralization using the antisera s608, s1207 and sE75 in the following manner: open bars: viruses were incubated with sera presenting persistent fraction (s1207 or sE75) at 1:5 dilution and subsequently incubated sera s608 at the same dilution; closed bars: first incubation with sera s608 and second with sera s1207 or sE75; grey bars: simultaneous virus incubation with competitors sera s608 and s1207 or sE75, both at 1:5 dilution. The figure shows the mean ± S.E. of the values of surviving fraction obtained in three independent experiments. (B) 100 PFU of the ASFV recombinant strain 1207VRΔTKβ-gal expressing the β-gal chromogenic marker gene were subjected to neutralization using the pool of antisera s608 and s1207 in the same manner as described in A. The figure shows virus plaques visualized through X-gal staining dye.

One of the most commonly accepted explanation for the apparition of the persistent virus fraction in neutralization assays is the emergence of antigenic escape mutants from the viral population (Hussain et al., 1987; Lambkin et al., 1994; Li et al., 1995; Watkins et al., 1993). To analyze this hypothesis in ASFV, 100 PFU of the 1207VR15 virus strain were subjected to a neutralization assay using the sE75 pool of sera. The surviving fraction profile obtained at different

antibody dilutions is shown in Fig. 2A, left chart, presenting similar results to that obtained in Fig. 1. Then, three viral plaques resistant to neutralization were isolated, resuspended in DMEM culture medium containing 10% FBS, sonicated and subjected to a second neutralization running. The neutralization profiles of these viruses with the same antibody are displayed in Fig. 2A, central chart, revealing a closed similarity to the profile obtained with the parental virus stock. Fig. 2 A, right chart, shows the result of a third neutralization running performed with three plaques (one plaque each), purified from the surviving viruses after the second neutralization running. Similar experiments were also carried out with s1207 pool of sera and viruses 608VR13 and 1207VR15 (data not shown), obtaining the same results in which it was not possible to purify neutralization escape mutants. These results strongly suggest that the persistent non-neutralizable virus fraction is not due to the emergence of antigenic escape mutants, since it was not able to enrich the non-neutralizable virus population after three neutralization runnings of non-neutralized virus clones.

Once discarded the generation of escape mutants, ASFV specific antibodies affinity and reversibility of virus-antibody complexes were studied to determine their relevance in the persistent virus fraction. Antibody affinity was studied by the existence of a time dependence for the resulting neutralization after virus-antibody complex formation, as described for other virus systems (Torfason et al., 1992). To study that possibility, 100 PFU of 1207VR15 virus were incubated during different times (2–24 h) with sE75, s1207 or s608 pools of sera at 1:5 dilution, prior to virus inoculation. Then virus-antibody complexes were analyzed for persistent fraction percentages on Vero cell cultures following the standard procedures. The results obtained are shown in Fig. 2B. There was not significant differences in the resulting surviving fraction by using different incubation times, suggesting that antibodies induced during infection neutralize the virus independently of the time of the complex formation.

As it has been proposed for other viruses (Suñé et al., 1990), the dilution of the virus-antibody complex after its formation could result in disaggregation and subsequent liberation of infective virus particles that may account for the persistent surviving fraction. To examine if this phenomenon occurs in ASFV neutralization assays, 100 PFU of 1207VR15 virus isolate were mixed with sera sE75, s1207 or s608 at 1:5 dilution and maintained at 37°C for 18 h. Then virus-antibody complexes were submitted at different dilutions just before inoculation to cell cultures. The results, shown in Fig. 2C, demonstrate that the dilution of the virus-antibody complexes did not increase the percentage of the surviving virus fraction observed with the undiluted sera, suggesting that the virus-antibody interaction is stable and independent of complex dilution.

In some virus models, it has been proposed the existence of non-neutralizing antibodies to specific virus antigenic determinants that could block the binding of those which neutralize the virus (Massey and Schochetman, 1981; O'Rourke et al., 1988). Those studies have been done by competition experiments using neutralizing and non-neutralizing monoclonal antibodies directed to proximal epitopes within the same antigenic virus protein. We carried out similar competition experiments to demonstrate the existence of blocking antibodies in some of the polyclonal antisera described above. Since these antisera were obtained from convalescent pigs inoculated with attenuated isolates, the experiments performed with these antibodies have the additional value of reflect a more closely related situation to that occurring during the immune response against the virus in infected pigs. The experiments were carried out on the basis that antibodies generated in pigs inoculated with the ASFV attenuated isolate 608VR13 are able to neutralize 100% of virus infectivity in in vitro (Fig. 1). This is a general feature of antibodies produced in pigs by this attenuated virus (Gómez-Puertas and Escribano, unpublished data). As shown in Fig. 1, the other two pools of sera generated by inoculation of the 1207VR15 and E75CV1-4 virus isolates (s1207 and sE75) showed a persistent surviving fraction.

Competition neutralization experiments of 608VR13 or 1207VR15 viruses were carried out using the three above mentioned pools of sera. As

a result of these competitions, we showed that preincubation of viruses (100 PFUs during 2 h) with s1207 or sE75 antisera inhibited the complete neutralization of the s608 serum which was added later and incubated for two additional hours (Fig. 3A, open bars). Simultaneous incubation of competitor pools of sera (s608 and s1207 or sE75) also rendered a persistent non-neutralized fraction (Fig. 3A, grey bars). Finally, competition neutralization experiments in which s608 sera was added prior to the other two competitor sera, rendered complete neutralization of the virus (Fig. 3A, closed bars).

These results indicate that when the virus is first incubated with incomplete neutralizing antisera, such as s1207 or sE75, critical epitopes in neutralization could be blocked by irrelevant antibodies. It suggests the induction in some cases of blocking antibodies during the immune response against ASFV in convalescent pigs. Complete virus neutralization mediated by serum s608 was not reversed after addition of a partial neutralizing serum. Additionally, simultaneous incubation with total and partial neutralizing antisera rendered a persistent virus fraction similar to that obtained with the sera containing incomplete neutralizing antibodies, suggesting that blocking antibodies are very efficient, probably because of their high affinity. To illustrate this phenomenon of blocking antibodies, the same experiment was done using the ASFV recombinant virus 1207VRΔTKβ-gal expressing the β-gal chromogenic marker gene (Gómez-Puertas et al., 1995) that allows the visualization of virus plaques by chromogenic dye staining with X-gal (Fig. 3B). Virus plaques were detected when incomplete neutralizing sera competed with s608 serum.

To rule out the possibility that other compounds present in sera, different to antibodies, might be the responsible for competition with neutralizing antibodies, we used a protein A column to purify antibodies from the pool of sera s1207. Purified antibodies were used in a conventional neutralization to determine the concentration of antibodies which reproduce the neutralization percentages obtained with the original serum. These purified antibodies reproduced the persistent virus fraction obtained with the parental serum and competed similarly with serum s608 (data not shown). It suggests that antibodies are the only responsible for inhibition of complete neutralization in competition assays.

The above data taken together allows us to suggest that the existence of a persistent surviving fraction in ASFV neutralization experiments could be due to the presence of non-neutralizing antibodies that are able to block the complete neutralization of some viral particles. The reason for the apparent absence of blocking antibodies in sera from convalescent pigs after inoculation with 608VR13 virus, which confers a very solid protection against this infection to the inoculated pigs, is unknown. Experiments to clarify this fact are in course. Presence of virus in organs from pigs inoculated with this virus could not be demonstrated, suggesting that this virus does not induce a persistent infection, as demonstrated with other viral strains (Carrillo et al., 1994).

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