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Review

Antibody-mediated neutralization of African swine fever virus: Myths and facts

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ABSTRACT

Almost all viruses can be neutralized by antibodies. However, there is some controversy about antibody-mediated neutralization of African swine fever virus (ASFV) with sera from convalescent pigs and about the protective relevance of antibodies in experimentally vaccinated pigs. At present, there is no vaccine available for this highly lethal and economically relevant virus and all classical attempts to generate a vaccine have been unsuccessful. This failure has been attributed, in part, to what many authors describe as the absence of neutralizing antibodies. The findings of some studies clearly contradict the paradigm of the impossibility to neutralize ASFV by means of monoclonal or polyclonal antibodies. This review discusses scientific evidence of these types of antibodies in convalescent and experimentally immunized animals, the nature of their specificity, the neutralization-mediated mechanisms demonstrated, and the potential relevance of antibodies in protection.

Contents

1. Introduction

African swine fever (ASF) is a viral disease that affects populations of wild boars and domestic pigs. In the latter it causes hemorrhagic fever, leading to high mortality rates and consequently significant economic losses. ASF is brought about by a large DNA virus, African swine fever virus (ASFV), which is to date the sole member of the family *Asfarviridae* (Dixon et al., 2011). However, the discovery of novel viral sequences in human serum and sewage has recently been reported. These sequences are clearly related to the asfarvirus family but are highly divergent from ASFV. The detection of these sequences suggests that asfarviruses show greater genetic diversity than previously thought and raises the possibility of human infection by these viruses (Loh et al., 2009).

There is no vaccine currently available to prevent ASF. Attempts to immunize animals using vaccine formulations prepared by conventional means and comprising infected cell extracts, supernatants of infected pig peripheral blood leukocytes, purified and inactivated virions, infected glutaraldehyde-fixed macrophages, or detergent-treated infected alveolar macrophages failed to induce protective immunity (Coggins, 1974; Forman et al., 1982; Mebus, 1988).

A number of recent reviews have addressed the role of cytotoxic T cells in immune defense against infectious agents; however, little attention has been devoted to an analogous role for humoral immunity, the component of the immune system that meets the microorganism at the portal of entry and interacts with it in all the body compartments that it subsequently invades. Regardless of the mechanism involved in virus inactivation, the attachment of antibodies to virus particles lead to loss of infectivity. However, the existence of non-neutralizing antibody, which binds to virus without diminishing infectivity, has long been recognized. There is a lack of basic knowledge about neutralization mechanisms in

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many animal viruses and about the relationship between *in vitro* neutralization and natural protection. A common misperception is that antibodies act solely by preventing the attachment of neutralized virus to target cells (Dimmock, 1993, 1995; Li et al., 1994). Antibodies, however, exert their neutralizing action by a variety of mechanisms, some of which are poorly understood. Neutralizing antibodies vary enormously in the rate and efficiency with which they act. The observation that neutralization rate constants can vary >1000-fold not only between distinct viruses but also between different antibodies to the same antigenic sites on the same virus suggests that the categorical capacity of an antibody or antiserum to neutralize is an over-simplistic correlate of protection (Krause et al., 1997). Moreover, individual virions may have many neutralizing epitopes, and in natural infections each of these may be available to bind to one of the variety of antibody specificities present at various concentrations, depending on the individual. Neutralization may be synergistic in such situations; however, it might also be antagonistic (i.e., the binding of antibody to one neutralizing epitope might interfere with the binding of antibody to another one) (Krause et al., 1997). In addition, some antibodies appear to up-regulate cell infection *in vitro*, a phenomenon referred to as antibody-dependent infection enhancement. Enhancement has been detected in many viral systems, usually with antibodies to envelope epitopes and typically with neutralizing antibodies (i.e., those that normally neutralize at adequate concentration) when bound at sub-neutralizing concentrations (Morens, 1994).

At present, it is widely accepted that nearly all viruses have neutralization sites. In the past, it was considered that only ASFV and Marburg and Ebola viruses lacked the ability to be neutralized by virus-specific antibodies (Dimmock, 1993). The last decade has revealed that filoviruses (Marburg and Ebola viruses) are efficiently neutralized by monoclonal and polyclonal antibodies (Hevey et al., 2003; Maruyama et al., 1999; Shedlock et al., 2010) and that these antibodies have protective properties (Hevey et al., 2003; Takada et al., 2007; Warfield et al., 2004). Early experiments with ASFV were structured and interpreted on the basis of the hypothesis that neutralizing antibodies are not induced by this virus (Hess, 1981; Viñuela, 1985).

Since the 80s, several strategies using viruses attenuated by passage in tissue culture (Gómez-Puertas et al., 1998; Ruiz Gonzalvo et al., 1986a,b) or using natural low virulence isolates (Boinas et al., 2004; Denyer et al., 2006; King et al., 2011; Leitao et al., 2001) have been developed to obtain pigs that are resistant to ASFV. These strategies allow study of the immune mechanisms responsible for protection. In ASF, several studies on the implication of antibodies in protection have convincingly demonstrated that they may delay onset of the disease and reduce viremia titers, two aspects that, to a great extent, determine the survival of pigs after infection. All of these issues will be discussed in this review.

2. Antibodies are relevant in the ASFV-induced protective immune response

Generally speaking, both cellular and humoral immune responses contribute to protection against viral infections and to the clearance of viruses from infected individuals. Specific experiments are required to discern the contribution of the mechanisms implicated in protection. Experimental vaccination protocols to obtain protected animals are the first step to analyze immune mechanisms, giving key information on which to develop in future strategies aimed at obtaining a licensed vaccine. In the case of ASF, only a few experimental models have been established (Gómez-Puertas et al., 1996; King et al., 2011; Leitao et al., 2001; Lewis et al., 2000; Oura et al., 2005; Ruiz Gonzalvo et al., 1986b; Stone et al., 1968; Zsak et al., 1993). Attenuated virus isolates OUR/T88/3 and E75CV$_1$-4 have been used to study cellular and humoral protective immune responses. Pigs exposed to OUR/T88/3 and then depleted of CD8+ lymphocytes were no longer fully protected from virus-related OUR/T88/1 challenge. This observation indicated that CD8+ lymphocytes play a critical role in the protective immune response to ASFV infection and that anti-ASFV antibody alone, from OUR/T88/3 infection, was not sufficient to protect pigs from OUR/T88/1 challenge. In contrast, antibodies obtained from pigs that had recovered from infection with one of the viruses most used in experimental protection experiments, denominated E75CV$_1$-4 virus, a Spanish strain (E75) adapted to grow in CV1 cells and propagated in pig macrophages (Barderas et al., 2001; Gómez-Puertas et al., 1996, 1998; Onisk et al., 1994; Ruiz Gonzalvo et al., 1986a,b) appeared to confer protection against ASFV infection. Sera from convalescent swine infected with this attenuated virus isolate neutralized the infectivity of virulent ASFV isolates E75, E70, Lisbon 60, Malawi Lil 20/1 and a low-passage tissue culture-adapted variant of E75, namely E75CV/V3, by 86–97% in Vero and macrophage cell cultures (Zsak et al., 1993). The role of anti-viral antibodies in homologous protective immunity to a virulent ASFV strain, E75, was examined by passive transfer experiments in swine. Eighty-five percent of the animals that received anti-ASFV immunoglobulin survived challenge infection. In contrast, 100% mortality was observed in the experimental group that received control immunoglobulin sera fractions or phosphate-buffered saline. With the exception of a significantly delayed and transient fever response, the animals who received anti-ASFV antibodies remained clinically normal following challenge, whereas the control group presented clinical ASF on day 4 post-challenge. In addition, a significant 3-day delay in the onset of viremia and a 10,000-fold reduction in both mean and maximum virus titers were observed for animals given anti-ASFV immunoglobulins. These results indicate that anti-ASFV antibodies alone protect swine from lethal infection with virulent ASFV (Onisk et al., 1994). Furthermore, they support the view that the antibody-mediated protective effect is an early event that effectively delays disease onset (Onisk et al., 1994). Other reports concerning the role of antibodies in ASFV protection showed that when these antibodies are transferred through colostrum, they also confer to suckling piglets a degree of protection against viral challenge (Schlafer et al., 1984a,b). Fig. 1 shows a schematic representation of *in vivo* antibody-mediated protection against ASFV described in the literature.

3. *In vitro* neutralization mechanisms of ASFV

Little work has been carried out 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 (Hess, 1981; Viñuela, 1985). However, other authors have demonstrated that a number of isolates of ASFV are neutralized by immune sera from convalescent swine and monoclonal antibodies (Borca et al., 1994; Gómez-Puertas et al., 1996; Ruiz Gonzalvo et al., 1986a,b; Zsak et al., 1993). Those studies that reported neutralization described a persistent fraction of non-neutralized virus of about 10%. This fraction was not demonstrable by an infection inhibition test, in which swine immune sera inhibited infection by the homologous and sometimes heterologous viruses in pig macrophages (Ruiz Gonzalvo et al., 1986b).

The use of conventional plaque reduction assays to measure antibody neutralization of ASFV presents frequent difficulties because many strains, especially low-passage viruses, show an absence or delay in plaque formation. Even more problems are encountered when performing neutralization assays in pig macrophages, the natural host cells. This would explain why, in neutralization assays, most authors use viruses that are highly adapted to cell lines. This point will be discussed further below because it has important consequences for the interpretation of

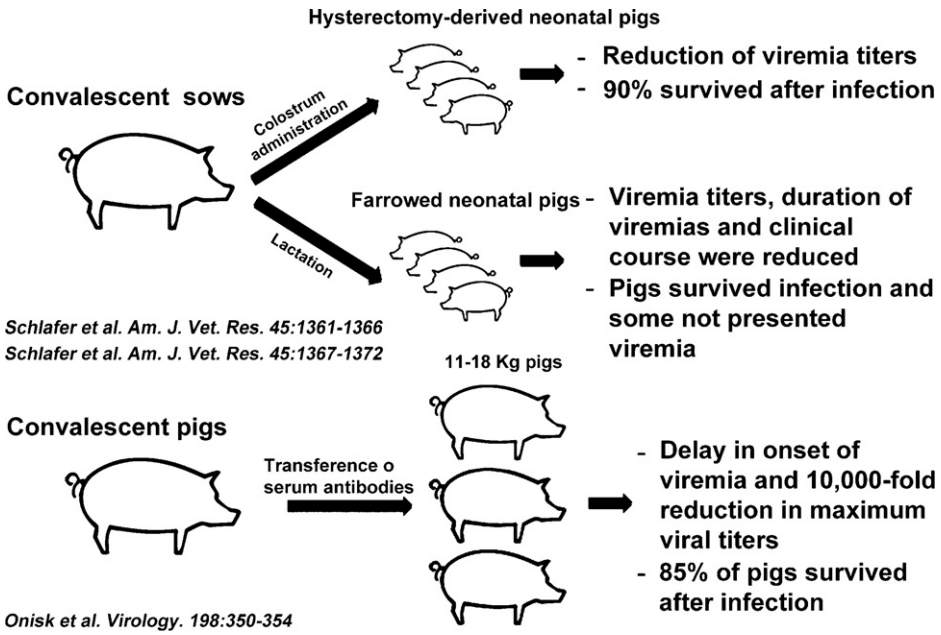

Fig. 1. Schematic representation of *in vivo* protection experiments described in the literature and involving antibodies taken from pigs that had recovered from ASF.

results. To overcome this problem, tests based on genetically modified viruses expressing chromogenic marker genes, such as β-glucuronidase or β-galactosidase, were developed (Gómez-Puertas et al., 1995) to facilitate in-depth studies of the antibody neutralization mechanisms of ASFV. Neutralization assays could now be performed with these viruses, either low- or non-passaged in cell culture, and experiments took less than a third of the time needed using non-recombinant viruses. In addition, small plaques could be detected more accurately by color contrast. These recombinant viruses also permitted differentiation by chromogenic staining of individual infected pig macrophages, thus facilitating the comparison of neutralization assays results in these primary cultures and cell lines.

The abovementioned recombinant viruses expressing marker genes have been used in studies that address the presence of neutralizing antibodies in convalescent pigs after infection with a range of attenuated viruses (Gómez-Puertas et al., 1996). Heat-inactivated sera from these pigs reduced the infectivity of several low cell culture-passage viruses between 87 and 100%. As previously mentioned, the persistent fraction of non-neutralized virus (4–13%) has been described (Ruiz Gonzalvo et al., 1986a; Zsak et al., 1993) and has also been found in other enveloped viruses (Ashe and Notkins, 1967; Jackson et al., 1991; Poumbourios et al., 1990). Neutralizing antibody induction seems to be an early event during infection with attenuated viruses as sera from convalescent pigs, in many cases, neutralized more than 50% of virus infectivity at day 9 post-infection. Neutralizing antibodies in vaccinated pigs reached a plateau at the end of the second week after infection (Gómez-Puertas et al., 1996).

Antibodies from pigs vaccinated with attenuated viruses use at least two mechanisms to neutralize virus infectivity in cell lines and macrophage primary cultures. Virus binding experiments to Vero cells or pig alveolar macrophages is a saturable mechanism (Alcami et al., 1989) and reached a plateau 120–240 min after the reaction was started (Gómez-Puertas et al., 1996). Antibody-mediated blocking of virus attachment to cells has been demonstrated experimentally by adding the antibodies before or after virus attachment to cells at 4 °C, a temperature at which virus internalization is inhibited (Fig. 2A). A relationship between inhibition of virus binding to susceptible cells and virus neutralization has been reported. Radiolabeled virus binding in the presence of anti-ASFV sera was inhibited by more than 80% at the time of maximum binding in the absence of neutralizing antibodies, both in Vero cells and in macrophages (Gómez-Puertas et al., 1996). Parallel neutralization experiments demonstrated that these sera reduce radiolabeled virus infectivity more than 90%. These results confirmed the existence of an ASFV neutralization mechanism in which antibodies inhibit the binding of the virus to cells.

A second ASFV neutralization mechanism has also been demonstrated by inhibiting radiolabeled virus internalization in the presence of immune sera in Vero cells or pig alveolar macrophages determining the detachment of non-internalized virus by proteinase K treatment (Fig. 2B). While the internalization of the virus previously attached to the cells at 4 °C was not affected by addition

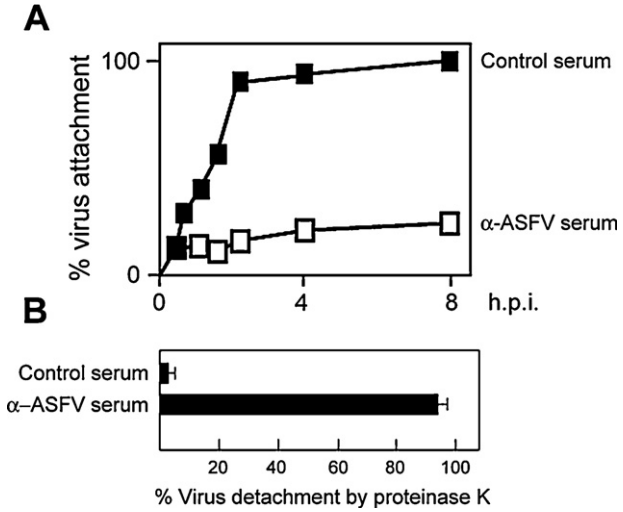

Fig. 2. Determination of ASFV neutralization mechanisms. (A) Percentage of inhibition of radiolabeled ASFV attachment to Vero cells after incubation with representative negative control or immune pig sera. (B) Percentage of radiolabeled ASFV detachment by proteinase K treatment of infected Vero cells with the virus at 4 °C (attached but not internalized) and then incubated with representative negative control or immune pig sera.

of a pre-immune control serum, 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 (Gómez-Puertas et al., 1996). No significant differences were observed between the experiments performed with Vero cells and those with pig macrophages. These observations revealed a second ASFV neutralization mechanism that abrogates a second step of the replication cycle involving virus internalization.

Experiments carried out with ASFV cannot rule out that one of these mechanisms may be predominant at different antibody-to-virus ratios, as described for other virus models (Sune et al., 1990). However, under the conditions described in this virus model, 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 (Gómez-Puertas et al., 1996). The combination of the two mechanisms neutralized more than 95% of virus infectivity. In summary, neutralization of ASFV may result from the inhibition of at least two steps of its replication cycle, namely virus attachment and internalization.

4. Factors influencing the susceptibility of ASFV to neutralization

In the past, it was considered that ASFV could not be neutralized by antibodies (Hess, 1981; Viñuela, 1985). Interestingly, a study published by Zsak et al. (1993) showed that serum from a convalescent swine to a particular ASFV isolate (E75) neutralizes the infectivity of various virulent ASFV isolates (E75, E70, Lisbon 60, Malawi Lil 20/1) and low-passage tissue culture-adapted variants by 86–97% in Vero and macrophage cell cultures. Unexpectedly, these immune sera failed to neutralize high-passage tissue culture-adapted ASFV variants, including Lisbon 60, Haiti, Dominican Republic I, Dominican Republic II, and Brazil II. A similar result was obtained with a neutralizing monoclonal antibody (135D4), which reacted with all viruses. On the basis of these findings, the authors proposed that tissue culture adaptation of ASFV isolates is related to the loss of specific determinants associated with virus neutralization.

ASFV propagation in cell lines has been shown to modify key genetic and phenotypic properties of the virus, such as replication capacity in pig macrophages, the natural host cell (Alcaraz et al., 1992; Rodriguez et al., 1994). However, dramatic changes in the antigenic conformation of several virus proteins involved in neutralization cannot be expected in all viruses propagated in cell lines. A later study (Gómez-Puertas et al., 1997) confirmed that low- and high-passage viruses differ in their susceptibility to neutralization (Fig. 3A). Those authors demonstrated that differences in susceptibility were not due to antigenic variability of critical epitopes and revealed the relevance of the phospholipid composition of ASFV virions; this composition was found to be distinct depending on the number of cell culture passages. A comparative analysis of phospholipid composition of the membranes of low- and high-passage viruses revealed differences in the relative amount of phosphatidylinositol in these two groups, independently of the cells in which the viruses were cultured (Fig. 3B). Further purification of low- and high-passage viruses by Percoll sedimentation showed differences in the phospholipid composition identical to those found with 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 phosphatidylinositol is a major phospholipid (Fig. 3C). In contrast, other phospholipids did not interfere with high-passage virus neutralization, suggesting that this membrane component is essential for correct epitope

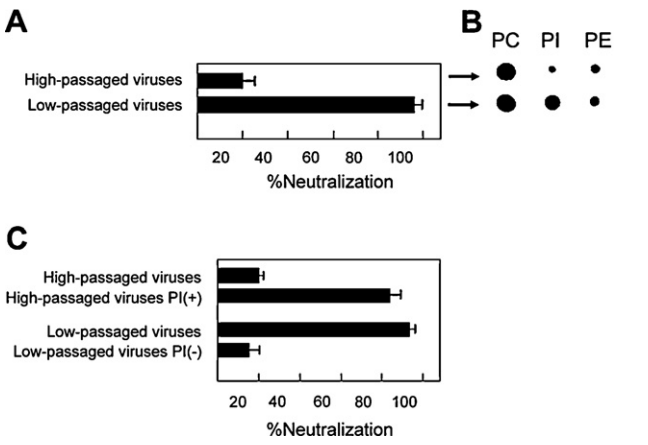

Fig. 3. Relevance of phospholipid composition of ASF virions for susceptibility to antibody neutralization. (A) Neutralization susceptibility of low- and high-passage ASFV isolates in cell lines, measured by a plaque number reduction assay in Vero cells and using sera from convalescent pigs.The figure shows the media and deviation standards of neutralization data obtained with three different viruses belonging to each group. (B) Phospholipid composition of viral membranes from representative purified low- and high-passage ASFV isolates. Differences are determined by the relative amounts of phosphatidylinositol incorporated. (C) Modification of neutralization susceptibility of low- and high-passage representative ASFV isolates after incorporation (+) or removal (−) of phosphatidylinositol from viral membranes.

presentation to neutralizing antibodies. In addition, the removal of phosphatidylinositol from a low-passage virus by a specific lipase transformed this virus from neutralizable to non-neutralizable (Fig. 3C). These data reveal the importance of the lipid composition of viral membranes for protein recognition by antibodies and may account in part for past difficulties in reproducibly demonstrating ASFV-neutralizing antibodies using high-passage viruses.

Given the differential neutralization patterns between high- and low-passage ASFVs, great care should be taken when selecting the virus isolate to be used in neutralization studies. Other examples of neutralization dependence on passage 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). As in other viral systems (Luan et al., 1995), ASFV directs the phospholipid composition of its membrane, since the relative percentages of phospholipids in the membranes of low- and high-passage viruses grown for several passages in the same cells differ (Gómez-Puertas et al., 1997). In Aleutian disease virus, the lipid composition of virions conditions their capacity to be neutralized (Stolze and Kaaden, 1987). The presence of high amounts of phosphatidylinositol in low-passage virions suggests that ASFV membranes are derived from the endoplasmic reticulum (ER) compartment of the host cell. This notion is supported by the phospholipid composition of newly formed virions, which is similar to the known membrane composition of the ER, where essentially all phosphatidylinositol is synthesized (van Meer, 1989). However, in contrast, high-passage, non-neutralizable virions have 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 the membrane formation mechanisms in low- and high-passage viruses differ (Sodeik et al., 1993).

Phospholipids are crucial for the antigenic properties of hepatitis B surface antigen (HBsAg). All epitopes of this protein defined by a panel of monoclonal antibodies show variation of reactivity after reconstitution with acidic phospholipids (Gómez-Gutierrez et al., 1994, 1995). Electrostatic interactions between HBsAg proteins and acidic phospholipids are partly responsible for the complete recovery of antigenic activity. Those authors proposed that the antigenic activity is dependent on the physical state of the phospholipid moiety. Once the conformation of the antigen in membranes is

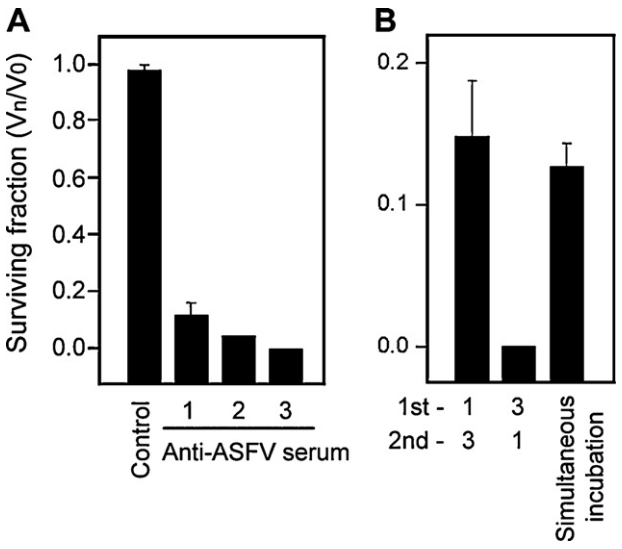

Fig. 4. Demonstration of the presence of neutralization blocking antibodies in sera from ASF convalescent pigs and the implication of these antibodies in a persistent non-neutralized virus fraction. (A) Determination of the persistent non-neutralized fraction of a representative ASFV isolate observed in neutralization assays using sera from convalescent pigs (1–3). Only serum number 3 neutralized 100% of virus infectivity. (B) Interference of antibodies present in a serum showing a typical persistent fraction with the complete neutralization of serum number 3 (serum number 1). The order used in the incubation of sera 1 and 3 with the virus determined the non-neutralized persistent virus fraction found at the end of the neutralization assays. The previous incubation of the virus with the partially neutralizing serum 1 abrogated the complete neutralization observed with serum number 3. This observation suggests the presence of blocking antibodies in serum 1.

established, additional interactions by the various phospholipids may alter the pattern of antigenicity, as shown with HBsAg (Gómez-Gutierrez et al., 1994, 1995) or with epitopes involved in ASFV neutralization.

5. The persistent non-neutralized virus fraction

A persistent non-neutralized ASFV fraction of about 10% is found with most convalescent swine sera in *in vitro* neutralization assays (Gómez-Puertas et al., 1996; Ruiz Gonzalvo et al., 1986a; Zsak et al., 1993) (Fig. 4A). The inability of this virus fraction 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.

In other viral models, this persistent fraction has been attributed to several factors, including the following: formation of viral aggregates that are non-accessible to antibodies (Taniguchi and Urasawa, 1987); incapacity of the sera to neutralize more than a few virus particles (low titer 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, thus the need of long incubations to achieve effective neutralization (Torfason et al., 1992); low stability of the antibody-virus complex, reversing easily after dilution (Sune et al., 1990); and the presence of blocking antibodies that inhibit virus neutralization (Massey and Schochetman, 1981; O'Rourke et al., 1988).

Most of these hypotheses have been studied in ASF using antisera from convalescent pigs infected with attenuated virus isolates and showing complete or incomplete virus neutralization (Gómez-Puertas and Escribano, 1997). Experiments revealed that incomplete neutralization of ASFV can be caused by virus aggregation (Zsak et al., 1993) but is not caused by low affinity or stability of virus-antibody complexes. Attempts to purify antigenic scape mutant viruses from the persistent non-neutralized ASFV fraction were also unsuccessful. Nevertheless, competition experiments between sera demonstrated that antibodies present in sera showing the persistent fraction abrogates the full neutralization (100%) mediated by other sera (Fig. 4B). These results strongly suggest that the induction of blocking antibodies during ASFV infection could be one of the main causes of the persistent surviving virus fraction observed in neutralization assays and could also explain the persistent infections observed in some convalescent pigs. However, it cannot be discarded that other aspects related to the virus infection, such as direct transmission of the virus cell to cell, could influence the scape of viruses to the antibody-mediated neutralization.

6. Proteins mediating the induction of ASFV-neutralizing antibodies

Complex viruses commonly have more than one outer protein which mediates neutralization, and this contributes to the complexity of neutralization (Dimmock, 1993). Often each protein has multiple neutralization sites and probably different mechanisms of neutralization. In addition, neutralization efficiency may differ between proteins. A monoclonal antibody, mAb-135D4, that recognizes the major capsid protein p72 exhibited strong ASFV neutralizing activity (Zsak et al., 1993). Consistent with the involvement of p72 in virus neutralization, immunoelectron microscopy, using mAb-135D4, located this protein on the surface of non-enveloped virus particles (Borca et al., 1994). By analysis of *in vitro*-translated products of the p72 gene specifically immunoprecipitated by mAb 135D4, a region between amino acid residues 400 and 404 was defined as necessary for the reactivity of this mAb. Five partially overlapping peptides (15mers) covering residues 388–446 failed to react with mAb 135D4, suggesting the conformational dependence of the epitope (Borca et al., 1994).

Sera from convalescent pigs revealed that p72, p30, and p54 were three of the most antigenic proteins during infection (Afonso et al., 1992; Alcaraz et al., 1990, 1995; Gómez-Puertas et al., 1996; Rodriguez et al., 1994, 1996). Pig immune sera against recombinant proteins p72, p30, and p54 neutralized more than 70% of virus infectivity (Gómez-Puertas et al., 1996). Surprisingly, antisera raised against recombinant protein p12 (Carrascosa et al., 1995) the viral attachment protein, did not reduce virus infectivity in an *in vitro* assay (Gómez-Puertas et al., 1996). Immunoelectron microscopy of ultrathin sections of ASFV-infected Vero cells using sera against these three proteins revealed their localization in mature intracellular viral particles (Gómez-Puertas et al., 1996). An analysis of the induction of the neutralizing antibodies mediated by these three proteins in the context of infection revealed that affinity-purified specific antibodies against these molecules, generated during infection, neutralized the virus in a similar way to monospecific antibodies obtained with recombinant proteins (Gómez-Puertas et al., 1996). Anti-p72 and -p54 sera neutralized the virus only before attachment to susceptible cells. However, serum recognizing p30 neutralized the virus equally when antibodies were incubated with the virus before or after attachment to cells (Gómez-Puertas et al., 1996). Therefore, we may conclude that antibodies against p72 and p54 inhibit a first step in the virus replication cycle related to virus attachment, while anti-p30 antibodies block a second step associated with virus internalization. These results correlate with the two neutralization mechanisms observed with sera from convalescent swine and allow the assignation of specific proteins to these mechanisms.

The role of p54 and p30 proteins in receptor-mediated ASFV endocytosis in swine macrophages has also been studied. These two proteins, which are released from ASFV particles after treatment of virions with a non-ionic detergent, bound to virus-sensitive

alveolar pig macrophages. This binding was found to be specifically inhibited by neutralizing antibodies obtained from a convalescent pig or from pigs immunized with recombinant p54 or p30 proteins (Gómez-Puertas et al., 1998). The binding of radiolabeled 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 presence of two distinct saturable binding sites for these proteins in susceptible cells. In addition, p54 blocked the specific binding of virus particles to the macrophage, while p30 impeded virus internalization. Both proteins independently prevented virus infection and in a dose-dependent manner, thereby suggesting that binding interactions mediated by these two molecules are necessary to give rise to a productive infection (Gómez-Puertas et al., 1998).

The relevance of the blockade of virus–cell interactions by p54 and p30 in the protective immune response to ASFV has also been addressed. Immunization of pigs with either recombinant p54 or p30 proteins induced neutralizing antibodies, which, as expected, inhibited virus attachment and internalization, respectively. However, immunized pigs were not protected against lethal infection and the course of the disease was not modified in these animals (only a delay in the disease onset was observed in p30-immunized pigs). In contrast, immunization with a combination of p54 and p30 proteins simultaneously stimulated both virus-neutralizing mechanisms and dramatically modified the course of the disease, rendering a variable degree of protection, ranging from a delay in the onset of the disease to complete protection against virus infection (Barderas et al., 2001; Gómez-Puertas et al., 1998). In an attempt to evaluate the role(s) of baculovirus-expressed p30, p54, p72, and p22 proteins from another pathogenic ASFV isolate (Pr4) in protective immunity, other authors immunized pigs with these proteins but failed to reproduce the degree of protection obtained previously (Neilan et al., 2004). Although ASFV-specific neutralizing antibodies were detected in the test group of animals, these exhibited only a 2-day delay to onset of clinical disease and reduced viremia levels at 2 days post-infection. However, by day 4 there was no significant difference in comparison to the non-immunized control group and animals died between 7 and 10 days after virus challenge. Differences in the immunization protocols or viruses used in experiments may determine the results obtained. Additional research is required to establish optimal immunization protocols and the true relevance of specific proteins in antibody-mediated protection against ASFV.

Finally, the baculovirus expressed hemagglutinin of ASFV, showing hemadsorption and erythrocyte-agglutinating activities, characteristic of the CD2 homolog protein induced by the virus in infected macrophages, triggered in immunized pigs hemagglutination-inhibition and temporary infection-inhibition antibodies. Interestingly, immunized pigs with this recombinant protein were protected against lethal infection (Ruiz-Gonzalvo et al., 1996). It opens new possibilities to incorporate more ASFV proteins in a potentially effective subunit vaccine formulation.

7. Antibodies directed to the dynein-binding domain of protein p54 neutralize ASFV

At very early infection stages, ASFV interacts with the 8 kDa light chain of cytoplasmic dynein (DLC8), a microtubular-based motor, through the structural virus protein p54. This interaction is critical during virus internalization and transport to factory sites. A 13 amino acid (aa) domain of p54 has been shown to be sufficient for binding to DLC8 (Alonso et al., 2001). Since the p54 dynein binding domain (DBD) has been identified and peptides with this sequence have been successfully used to inhibit virus infection in cell cultures (Hernáez et al., 2010), we explored how antibodies against this viral aa sequence interfere with ASFV infection.

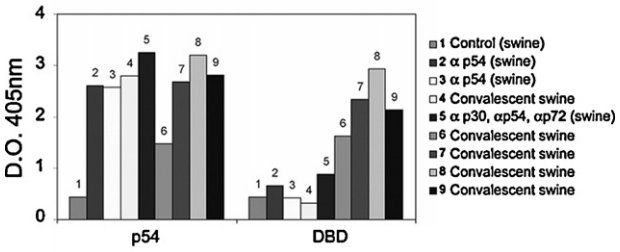

Fig. 5. Determination by ELISA of antibodies recognizing the dynein binding domain (DBD) of ASFV in sera from recovered pigs after infection (sera 6–9) or immunized with protein p54 (sera 2–5). ELISA plates were coated with purified *E. coli*-derived p54 protein or with a peptide representing the DBD sequence.

In that study, we first determined the immunological dominance of the DBD aa sequence in the context of infection. The specific antibody response against the DBD sequence was evaluated by ELISA, using synthetic peptides representing the DBD of ASFV, in sera from pigs recovered from experimental virus inoculations. Additionally, we also analyzed sera from pigs immunized with recombinant p54 (Fig. 5). Independently of the antibody titer of each serum analyzed, all sera tested recognized the p54 protein, but only sera from recovered pigs reacted in ELISA with the peptides belonging to the DBD. This finding indicates that virus replication, which is normally required to achieve protection against ASFV, is needed to generate specific antibodies against the DBD sequence.

To test the potential of antibodies directed to DBD to inhibit infection, we generated specific antibodies against this aa sequence. Since peptides are usually of low immunogenicity, we fused the encoding sequence for DBD to *Salmonella typhimurium* type 2 (STF2) flagellin, a TLR5 ligand that promotes strong antibody responses in

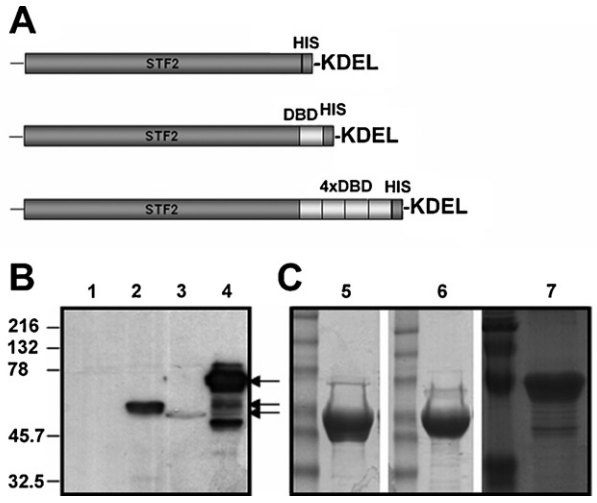

Fig. 6. Expression of flagellin fusion proteins containing the DBD ASFV sequence by the baculovirus expression system. (A) Schematic representation of STF2, STF2-DBD and STF2-4xDBD constructs cloned in a baculovirus vector. All 3 constructs were designed with His-tag and KDEL sequence and were expressed in a baculovirus expression system (Bac to Bac®; Invitrogen). (B) Detection of the recombinant proteins STF2-DBD (2), STF2 (3), and STF2-4xDBD (4), produced in infected Sf21 insect cells, by Western blot with a monoclonal antibody anti-His-tag. A protein extract from insect cells infected with the wild-type baculovirus without an insert was included as a negative control (1). Arrows indicate the position of the recombinant proteins. (C) Purified recombinant proteins obtained by IBES® technology (*T. ni* insects as living biofactories); STF2 (5), STF2-DBD (6), and STF2-4xDBD (7), were observed by Coomassie brilliant blue staining of SDS–PAGE gels.

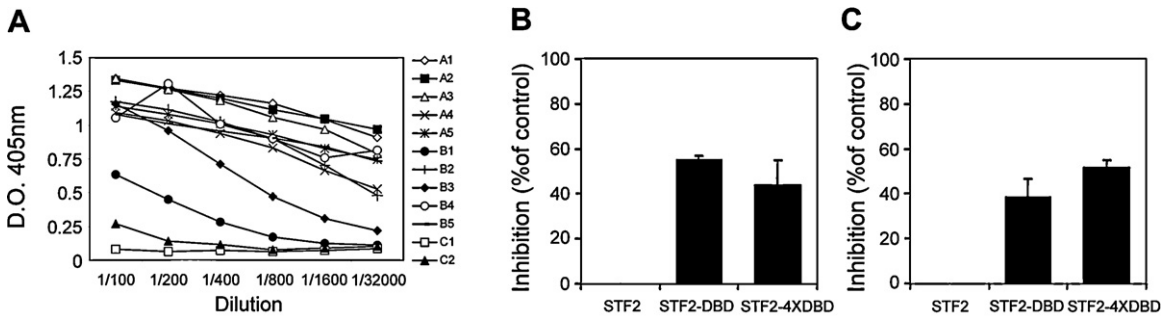

Fig. 7. (A) Antibody immune response against several recombinant flagellin-fusion proteins in immunized mice. The antibody response was measured by ELISA plates coated with purified *E. coli*-derived p54 protein. Sera were tested at a range of dilutions; Protein STF2 (C1 and C2), STF2-DBD (B1–B5) and STF2-4xDBD (A1–A5). Neutralization values obtained with the sera from mice immunized with the flagellin-DBD fusion construct using the virus isolate 1207 VR19 (B) or 608 VR14 (C). Neutralization analyses were performed in Vero cells by a plaque number reduction assay using 100 PFU of the different viruses.

immunizations (Medzhitov et al., 1997). This strategy was successfully used previously to raise antibodies against this sequence as a peptide model (Terrón-Expósito et al., 2012). The ORF of STF2 protein was cloned into Pfastbac (Invitrogen) using *Salmonella typhimurium* DNA as a template. The recombinant plasmid-generated pFB-STF2-H-KDEL contained the KDEL sequence and 10 histidine residues, which facilitated the expression and purification of the protein respectively. The DLC8 binding domain found in the ASFV p54 protein was cloned into pFB-STF2-H-KDEL, in frame with STF2, to generate the pFB-STF2-DBD-H-KDEL plasmid. The pFB-STF2-4xDBD-H-KDEL vector, encoding four copies of STF2, was constructed by cloning the PCR fragments in tandem into pFB-STF2-H-KDEL (Fig. 6A). In order to optimize protein expression, we verified the production of recombinant proteins in sf21 cells prior to the infection of *Trichoplusia ni* insect larvae (IBES® technology). Total soluble protein fractions were extracted from insect larvae and processed as described previously (Pérez-Filgueira et al., 2006). Expression was analyzed by Western blot with a mAb against the histidine tag. The antibody recognized specific bands corresponding to recombinant proteins STF2, STF2-DBD and STF2-4xDBD at the expected electrophoretic mobility (Fig. 6B). Proteins obtained from inoculated larvae were purified under native conditions and identified in a Coomassie blue-stained gel (Fig. 6C).

Purified STF2-DBD fusion proteins were used to immunize mice. Each mouse received 50 μg of protein per inoculation in Freund's complete (first dose) or incomplete adjuvant (second and third dose). Fifteen days after the last injection, polyclonal serum was prepared from defibrinated blood and titrated by ELISA. The immune response to the recombinant STF2-4xDBD was strong in all animals ($n = 5$). In the case of the recombinant fusion protein containing one copy of the DBD sequence, the amount of antibody produced varied from mouse to mouse, with 3 animals with the same response than obtained with constructs containing four copies of DBD and 2 animals with a reduced response (Fig. 7A).

Sera from each group were tested for non-complement-mediated virus neutralizing activity in Vero cell monolayers in a focus reduction neutralization test. Immune mice sera, raised to one or four copies of the DBD, reduced the virus infection plaques up to 61.5%, as determined by immunofluorescence (Fig. 7B and C). Foci, counted in duplicate wells, were averaged and compared to those obtained with neutralization assays performed with pre-immune sera. Sera obtained against STF2 did not show any neutralizing activity. On the basis of the above results, we may conclude that the p54 DBD is implicated in virus neutralization mediated by antibodies. However, further experiments are needed to determine how the induction of antibodies against this domain modifies the course of the disease in vaccinated pigs.

8. Concluding remarks

Despite original reports indicating the lack of neutralizing activity of sera from animals infected with ASFV, overwhelming evidence of neutralizing antibodies against this virus has been provided by numerous laboratories in the last 15 years. Moreover, several elegant experiments have revealed the relevance of antibodies in protection against this fatal disease. However, in terms of antibody-mediated neutralization, ASFV has uncommon particularities that are shared by other viruses. Several of these singularities may explain why some authors have concluded that ASFV does not induce neutralizing antibodies in pigs that have recovered from infection. The peculiarities of ASFV include loss of susceptibility to neutralization by cell culture passage as a result of changes in the phospholipid composition of viral membranes and/or the presence of sera blocking antibodies that inhibit complete neutralization. However, a number of ASFV proteins have been undoubtedly implicated in the induction of neutralizing antibodies during infection and assigned to one of the two neutralization mechanisms described for this virus. In addition, some critical epitopes in neutralization have also been characterized in proteins p72 and p54. Antibody-mediated neutralization is a key defense mechanism against viral infections. Although cell-mediated immune mechanisms may make a significant contribution to protection against ASFV, as in other viruses that infect macrophages, current data encourage us to explore vaccine formulations with the aim to maximize the induction of critical and potent neutralizing antibodies. Strategies to stimulate neutralizing antibodies should be considered feasible in the design of ASF vaccine programs.

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