Recent advances in the development of recombinant vaccines against classical swine fever virus: Cellular responses also play a role in protection

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Abstract

Classical swine fever virus (CSFV) is the causative agent of one of the most devastating porcine haemorrhagic viral diseases, classical swine fever (CSF). CSFV mainly infects endothelial cells and macrophages and at the same time promotes bystander apoptosis of the surrounding T cells, causing strong immune suppression and high mortality rates. Most animals experience acute infection, during which they either die or survive by producing neutralising antibodies to the virus. However, in a few cases, the impaired immune system cannot control viral progression, leading to chronic infection.

Efficient live attenuated vaccines against CSFV exist and are routinely used only in endemic countries. The ability of these vaccines to replicate in the host, even at very low rates, makes it extremely difficult to distinguish vaccinated from infected animals, favouring a restricted policy regarding vaccination against CSFV in non-endemic countries. There is a clear need for efficient and safer marker vaccines to assist in the control of future CSF outbreaks. In this review article, some of the most recent advances in the field of recombinant vaccines against CSFV are presented and the nature of the protective immune responses they induce is discussed.

Keywords: CSFV; Vaccine; Neutralising antibodies; T cell response; Immune suppression

Introduction

Classical swine fever (CSF) or hog cholera is a highly infectious viral disease included in the list of diseases notifiable to the OIE (www.oie.int). It affects domestic and wild pigs and is considered to be one of the most devastating diseases for the pig industry throughout the world from both the economic and sanitary point of view (Moennig et al., 2003).

In vivo, CSF virus (CSFV), the aetiological agent of CSF, targets the porcine immune system, mainly those cells derived from the monocyte-macrophage lineage, inducing marked bystander apoptosis of non-infected surrounding lymphocytes, by mechanisms still not totally understood (Summerfield et al., 1998a, 2001). Highly virulent strains of CSFV cause marked immune suppression and high mortality (Susa et al., 1992; Lee et al., 1999; Gomez-Villamandos et al., 2003). The clinical signs of the disease depend not only on the specific strain of the virus, but also on many other factors, such as the age and the immune status of the animals (Moennig et al., 2003).
The global distribution of CSF has been extensively reviewed (Frias-Lepoureau and Greiser-Wilke, 2002; Greiser-Wilke and Moennig, 2004; Dong and Chen, 2007). The disease is endemic in Asia and is prevalent in many countries of central and South America. Several outbreaks have been reported in Caribbean countries in recent years (Frias-Lepoureau, 2002; de Arce et al., 2005; Pereda et al., 2005). Little is known about the situation in Africa, where CSF has been reported in Madagascar, and recently in South Africa (Sandvik et al., 2005).

While CSF was eradicated from North America several decades ago, a progressive eradication programme has been implemented in the European Union (EU) since the early 1990s. This programme is based on a non-vaccination policy, the culling of infected animals or those in contact with infected herds (stamping out) and the restriction of animal movements or their products. Vaccination is only allowed in emergencies (European Union, 1980). However, in spite of control programmes, the virus has been introduced periodically into the EU via wild pigs or foreign imports, as occurred during the 1990s in Belgium, Germany, The Netherlands, Spain and Italy and, since 2000, in the UK, Spain and Germany (Paton and Greiser-Wilke, 2003; Dong and Chen, 2007).

As with many other diseases affecting livestock, the most efficient vaccines currently available against CSFV are live attenuated vaccines that were developed more than 50 years ago (de Smit et al., 2000). Although guaranteeing high protection rates, the live attenuated vaccines against CSF have some disadvantages. For example, vaccination with live attenuated viruses elicits similar antibody patterns to those observed in naturally infected animals, making it extremely difficult to differentiate vaccinated animals from infected ones. Recent advances in vaccination strategies have circumvented this dilemma by the use marker or DIVA (differentiation of infected from vaccinated animals) vaccines (van Oirschot, 1999; de Smit, 2000).

Despite recent efforts to develop new and safer marker vaccines against CSFV, along with improved diagnostic tools, there is still a need for further improvements (Floegel-Niesmann, 2001; Greiser-Wilke and Moennig, 2004). This review covers some of the most relevant advances in this field. However, further efforts to understand the immune mechanisms relevant to CSFV protection and to improve diagnostic tools are required to develop safer marker vaccines against this important disease.

**Immune response induced by CSFV infection in pigs**

Understanding the interaction of CSFV with the cells of the porcine immune system and its role in viral pathogenesis is a key point in designing new antiviral strategies. Work carried out several decades ago described the capacity of CSFV to destroy the lymphoid follicles (Cheville and Mengeling, 1969) and these results have subsequently been confirmed (Pauly et al., 1998). Among the main targets for CSFV are bone marrow cells from the monocyte-macrophage lineage (SWC3⁺; SWC8⁺). Interestingly, while mature granulocytes (SWC3⁺; SWC8⁺) are not susceptible to CSFV infection, the virus does infect the less differentiated myeloid progenitor cells (SWC3low⁺; SWC8⁺), thus explaining the presence of CSFV in peripheral blood mature SWC8⁺ cells (Summerfield et al., 1998a, 2001).

Despite the high mortality and the severity of lesions in animals with virulent CSFV, one peculiarity of CSFV infection is that most infected cells in the bone marrow remain healthy, while surrounding uninfected cells succumb, mainly due to bystander apoptosis. This is most probably due to soluble factors secreted by the infected cells (Summerfield et al., 2001).

Infected pigs show marked immune suppression, with an altered population of T cells and depletion of lymphocytes (van Oirschot et al., 1983), mainly CD4⁺ and CD8⁺ T cells. Depending on the virulence of the viral strain, pigs can have as much as 90% of their total T cells depleted in the final stages of the disease (Pauly et al., 1998). This effect can be observed as early as one day after infection, even before viraemia has been established (Summerfield et al., 1998b). Immunosuppression can be detected much earlier than seroconversion and clinical signs of the disease, which is relevant both for early diagnosis and for the study of viral pathogenesis (Pauly et al., 1998; Summerfield et al., 1998a, 2000; Ganges et al., 2005).

As with many other severe haemorrhagic diseases, CSFV infects endothelial cells (Avirutnan et al., 1998; Yang et al., 1998). These cells seem to play a key role in CSFV pathogenesis, which is characterised by the development of microthrombi, disseminated intravascular coagulation and fibrinolysis, among other clinical signs (Summerfield et al., 2000). While CSFV infection of endothelial cells suppresses both interferon production and apoptosis, it induces inflammatory cytokines (Campos et al., 2004).

CSFV exploits the migratory capacity of macrophages and dendritic cells (DCs) for dissemination throughout the body (Carrasco et al., 2004). Activation of anti-apoptotic pathways in infected cells ensures virus survival, whereas induction of the expression of soluble pro-inflammatory cytokines promotes bystander apoptosis of the surrounding T cells, thus avoiding recognition of the infected cells (Bensaude et al., 2004).

Recent evidence suggests that CSFV might induce immune suppression not only by secreting cytokines, but also by other mechanisms, such as expressing the CSFV structural E₃R protein, which has been shown to be toxic for T cell lymphocytes in vitro (Bruschke et al., 1997; Pauly et al., 1998). CSFV also activates T cells that secrete cytokines, such as IL10, which is probably a key cytokine in the immunosuppression observed after CSF infection (Suradhat et al., 2005). Considering the capacity of CSFV to escape the immune response, strategies must be designed to control the infection and to prevent CSFV-mediated immune evasion.
As mentioned above, CSF is generally a devastating disease characterised by high mortality rates. It induces a marked immune suppression in infected individuals that, depending on the virulence of the strain and the immunological status of the infected animal, might cause the death of the animals before an immune response is induced. However, some animals are able to overcome infection as a result of protective immune responses, becoming protected from re-infection for the rest of their lives (van Oirschot, 2003a).

In some cases, the immune responses mounted are not sufficient to clear the virus from the body, leading to persistence of CSFV in the host. Chronic infections can be established in the presence of neutralising antibodies (Mengeling and Packer, 1969). Conversely, congenital infections are not neutralising antibodies, which do not develop specific antibodies against the virus (Van Oirschot, 1979; de Smit et al., 2000). This is probably due to immunotolerance developed during foetal lymphocyte differentiation (Liess, 1988). Persistently infected animals continuously shed virus and are a potential source of new CSF outbreaks (Vannier et al., 1981; Carbrety, 1989), as well as creating problems in diagnosis.

Persistence of CSFV in the animal and the induction of chronic infection makes it necessary to understand not only how the virus interacts with the cells of immune system, but also the specific immune responses that are involved in protection (Moennig, 2000). Recent reports indicate that infected pigs are able to induce both specific CD4+ T cells and cytotoxic T lymphocyte (CTL) responses (CD8+ T cells) from 1 to 3 weeks after infection, even before neutralising antibodies appear. The specific CTLs mainly recognise the structural E2 protein and the non-structural NS3 protein of CSFV (Ceppi et al., 2005; Ganges et al., 2005; Rau et al., 2006). These results correlate with previous findings describing a CTL epitope at the interface between the NS3 and NS4 viral proteins (Pauly et al., 1995). Several swine leucocyte antigen (SLA) types I and II peptides have been characterised, most of them mapping to the NS3 polypeptide and to the structural E2 protein (Armengol et al., 2002).

Neutralising antibodies in pigs surviving CSFV infection cannot be detected until 2–4 weeks post-infection and they remain relatively constant for the rest of the life (van Oirschot, 2003a). Most neutralising antibodies specifically recognise the structural E2 and E3 proteins (Paton et al., 1991; Weiland et al., 1992; Konig et al., 1995) and their presence has been associated with protection (Rumenapf et al., 1991). Antibodies against the NS3 protein are also induced upon infection but, despite their ability to recognise different pestiviruses, they are non-neutralising antibodies (Greiser-Wilke et al., 1992). The E2 glycoprotein is the most immunogenic CSFV protein and the only one capable of conferring protection against CSFV challenge by itself (Rumenapf et al., 1991; van Zijl et al., 1991; Hulst et al., 1993). Thus, E2 has become the main candidate for inclusion in subunit and recombinant vaccines against CSFV.

**Vaccine strategies against CSFV**

**CSFV classical vaccines**

While the early inactivated vaccines against CSFV were not effective against virus infection (Koenen et al., 1998), live attenuated vaccines were demonstrated to be very efficient at preventing disease. The first immunisation experiments with live attenuated lapinised strains (attenuated after serial passages in rabbits) were performed over 60 years ago (Koprowski et al., 1946). Since then, little progress has been made in this field. CSFV attenuated vaccines are safe, induce high levels of neutralising antibodies and are capable of protecting against highly virulent strains of CSFV, even in pregnant sows as early as 5 days after vaccination. Attenuated CSFV vaccines remain the method of choice to control the disease, especially in areas in which CSF remains enzootic.

The mechanisms mediating the protection conferred by attenuated strains of CSFV are not fully understood. Vaccinated animals are protected against disease before neutralising antibodies are detected (Suradhat et al., 2001). After vaccination with the C strain, antibodies are detectable as early as 12 days post-immunisation, reaching a peak at 4–12 weeks (Precausta et al., 1983; Terpstra et al., 1990). Antibodies can persist for life in animals that have received a single dose of vaccine (Terpstra and Tielman, 1976).

Neutralising antibodies play an important role in protection against CSFV (Terpstra, 1977; Van Bekkum, 1977; Biront et al., 1987). However, some reports indicate a lack of complete correlation between antibody titres and protection (Terpstra, 1977; Van Bekkum, 1977) and therefore other arms of the immune response might also be important in conferring protection. As a general rule, animals developing neutralising antibodies will be protected against subsequent challenge (Terpstra and Wensvoort, 1988; Suradhat et al., 2001), but protection after vaccination in the absence of neutralising antibodies also has been observed (Suradhat et al., 2001; van Oirschot, 2003b; Ganges et al., 2005).

Although classical live attenuated vaccines against CSFV have been shown to be effective, they also have several disadvantages. Antibodies induced upon vaccination are indistinguishable from those elicited in natural infections, which impairs their differential diagnosis (Moennig, 2000). Also, errors in the conservation and/or manipulation of the vaccines and incomplete coverage of the overall susceptible population with the vaccine, might lead to protection failures and to the appearance of carrier animals that cannot be distinguished with the current diagnostic tools (Leunen and Strobbe, 1977; Chenut et al., 1999; Kaden et al., 2002). In addition, attenuated vaccines are able to replicate to a limited degree in lymphoid organs,
mainly in the tonsils, for at least 15 days, and are indistinguishable from the virus in the field using standard diagnostic tests (Lubroth, 1999; Kaden et al., 2004).

Vaccination against CSFV currently is not permitted in the EU, except in emergency outbreaks. The strict limitations and restrictions of the animal movement policy make further implementation of vaccination difficult, thus favouring the culling of animals. Apart from ethical reasons, the slaughter of non-infected animals in infected control zones has caused major economic losses in affected countries in the EU. Therefore, the policy of non-vaccination or limited emergency vaccination may not to be sustainable. Clearly, new strategies have to be implemented to control CSF, avoiding the sacrifice of large numbers of pigs if an outbreak occurs within EU borders (Mangen et al., 2001; Paton and Greiser-Wilke, 2003).

**New vaccines against CSFV**

As for many other viruses, the generation and use of infectious clones has provided new opportunities for understanding and characterising mechanisms of viral replication and pathogenesis (Risatti et al., 2005). Infectious clones of attenuated strain C, Alfort/Tübingen, Eysruper and Brescia strains of CSFV have allowed the determination of E\textsuperscript{rms}, N\textsuperscript{pre} and E2 as potential virulence factors (Meyers et al., 1999; Mayer et al., 2004; van Gennip et al., 2004; Risatti et al., 2005).

Information generated using reverse genetics has facilitated further the identification of CSFV proteins or protein domains that determine viral virulence and host range, as well as the design and development of new rational vaccines against CSF. The observation that virulent CSFV strains in the field can evolve into more attenuated forms of the virus under certain epidemiological conditions has opened new avenues to study specific changes in the viral genome involved in virulence, particularly in the E2 gene (de Arce et al., 2005).

It has been possible to generate deletion mutant viruses lacking the entire E2 or E\textsuperscript{rms}. These deletion mutants have been shown to be safe vaccines, due to their ability to grow only in competent cell lines that provide the deleted protein in trans. Upon in vivo injection, the mutant viruses induced strong immune responses without producing new viral progeny (Widjojoatmodjo et al., 2000). Similar strategies have been used to develop several chimaeras by exchanging the E2 or the E\textsuperscript{rms} proteins from the attenuated C strain of CSFV with those from antigenically related pestiviruses (van Gennip et al., 1991; Hahn napf et al., 1991; Hammond et al., 2000, 2001), or species-specific vectors, such as porcine poxviruses or Aujeszky's disease virus (van Zijl et al., 1991; Hahn napf et al., 1991). In all cases, these recombinant vectors have conferred protection against clinical disease.

Bovine viral diarrhoea virus (BVDV), a pestivirus antigenically related to CSFV, has been used as a vector to express the CSFV E2 glycoprotein, inducing protection against CSFV challenge (Reimann et al., 2004). Despite the protective potential of these vaccines, much remains to be done to characterise the immune responses they induce and their protective efficacy in terms of viral dissemination and transplacental transmission of CSFV (Reimann et al., 2004).

**Subunit vaccines: immunogenicity of the CSFV E2 glycoprotein**

Several strategies have been developed to generate DIVA vaccines and accompanying diagnostic tests in order to fulfil the need for new and safer “marker vaccines” to control CSF (van Oirschot, 1999). The most efficient subunit vaccines developed against CSFV to date are based on the E2 major glycoprotein from the CSFV envelope. A variety of degrees of protection have been afforded by using different forms of the recombinant E2 glycoprotein expressed in the baculovirus system (Hulst et al., 1993; van Rijn et al., 1996; Bouma et al., 1999). One of the most immunogenic versions of the recombinant E2 glycoprotein lacks the transmembrane domain, thus facilitating its secretion and purification from supernatant of baculovirus infected cells.

So far, most of E2 subunit marker vaccine formulations are in the experimental phase and only two vaccines licensed by the European Agency for the Evaluation of Medicinal Products (EMEA) are commercially available (Bayovac CSF, Bayer; Porcilis Pestis, Intervet). Vaccinated animals develop specific antibodies that exclusively recognise the E2 glycoprotein, whereas pigs infected with field strains of CSFV also develop antibodies against the E\textsuperscript{rms} protein, which can be distinguished with a specific enzyme-linked immunosorbent assay (ELISA) (de Smit et al., 2001). Despite the advantages of using subunit vaccines, their protective efficacy is still under evaluation (Paton and Greiser-Wilke, 2003; Greiser-Wilke and Moennig, 2004).
Compared with classical attenuated vaccines, the protection conferred with subunit vaccines is much more limited, especially among pregnant sows, and there is a higher risk of establishment of persistently infected individuals (Ahrens et al., 2000; Depner et al., 2001; de Smit et al., 2001; Paton and Greiser-Wilke, 2003; van Oirschot, 2003b; Greiser-Wilke and Moennig, 2004; Vannier, 2004).

Two final reasons, both related to the lack of efficient differential diagnostic methods, have contributed to the non-implementation of these vaccine strategies as an emergency measure in the field (Vannier, 2004). Firstly, specific antibodies against E<sub>ns</sub> are not developed until 3–6 weeks after natural infection with field strains of CSFV (depending on the virulence of the CSFV strain), which makes the differential diagnosis at early stages of infection difficult (de Smit et al., 2000). Secondly, the available E<sub>ns</sub> based ELISAs are still far from being as sensitive and specific as the E2 based ELISAs (Greiser-Wilke and Moennig, 2004).

Using the baculovirus expression system, it has been possible to map protective domains within the E2 glycoprotein (van Rijn et al., 1996, 1999) and, more recently, to obtain a detailed characterisation of some protective epitopes within E2 (Dong et al., 2005; Dong and Chen, 2006). All these studies now would permit the inclusion of defined neutralising B cell epitopes in new recombinant vaccines, with the aim of inducing protective immune responses that facilitate the differential diagnosis of vaccinated versus CSFV infected individuals.

In addition to the development of new, improved CSF vaccines, parallel efforts should be made to develop new diagnostic methods capable of differentiating vaccinated from infected individuals. Unfortunately, as for many other diseases, commercial interests might have contributed to the slow development of new and more efficient diagnostic methods and vaccines against CSFV (Greiser-Wilke and Moennig, 2004).

The E2 based vaccines show limitations similar to those found for other CSF vaccine strategies. Some animals do not produce specific CSFV antibodies in response to vaccination and there is not a complete correlation between protection and the induction of neutralising antibodies. Research on new DIVA vaccines capable of conferring high levels of protection against CSFV should be complemented by research on the development of new diagnostic tools (van Oirschot, 2003a).

**DNA vaccines against CSFV: a real alternative?**

DNA immunisation is one of the most promising strategies of vaccination developed in the last decade. DNA vaccines are capable of inducing both humoral and cellular immune responses. They are easy to produce and have been demonstrated to be safe, even for immunisation of neonates in the presence of maternal antibodies (Hassett et al., 1997; Manickan et al., 1997). Due to its simplicity, DNA vaccination also permits customised design of vaccines (Doria-Rose and Haigwood, 2003) and has the potential to direct encoded antigens to specific pathways (Rodriguez and Whitton, 2000; Ertl and Thomsen, 2003; Leifert et al., 2004). DNA immunisation can be a useful tool in understanding the intrinsic immunological mechanisms involved in protection against a given pathogen (Sedegah et al., 1994; Learmonth et al., 2003) or to map immunogenic protective determinants (Rodriguez et al., 2001).

CSFV has been included in this revolution within the field of vaccine development. Most of the DNA vaccines developed against CSFV are based on the expression of its major immunogenic determinant, the E2 glycoprotein, from different viral strains. Some authors report the use of DNA vaccines encoding the full-length E2 glycoprotein, while others use mutant forms lacking its transmembrane domain (Andrew et al., 2000; Markowska-Daniel et al., 2001; Yu et al., 2001).

Comparison of the results obtained in vaccination and challenge studies by different groups using DNA vaccines against CSFV is difficult due to different forms of E2, different doses of challenge virus and differences in the virulence of challenge strains of CSFV. Even though immunisation with large amounts of a DNA vaccine encoding the entire E2 glycoprotein (including its transmembrane region) was capable of protecting pigs from clinical CSF, no direct correlation was observed with the presence of neutralising antibodies against CSFV (Andrew et al., 2000). Similar levels of protection have been observed by using prime-boost protocols of vaccination with DNA plasmids followed by injection of recombinant adenovirus expressing the entire CSFV E2 protein (Hammond et al., 2001).

Protection against CSFV in the absence of neutralising antibodies has been demonstrated with an experimental DNA vaccine encoding the full-length CSFV E2 glycoprotein (Ganges et al., 2005). Although no antibodies were detectable prior to CSFV challenge, an accelerated humoral response was observed in some of the vaccinated animals, with neutralising antibodies being detectable as early as at day 6 post-infection. Interestingly, lack of viraemia, protection from clinical disease and protection from immunosuppression correlated with the induction of both neutralising antibodies and major histocompatibility complex (MHC) class II restricted cellular responses against CSFV (Ganges et al., 2005). This suggests that other arms of the immune response, such as the specific induction of MHC class II restricted CD4<sup>+</sup> T cells, may play a role in protection against CSFV.

An ideal vaccine against CSFV therefore should induce both humoral and cellular responses. Recently, it has been shown that the induction of neutralising antibodies can be improved after DNA immunisation by co-injecting several cytokines, such as IL3 (Andrew et al., 2006) and IL8 plus the porcine cell surface molecule CD154 (Wienhold et al., 2005).
Conclusions

Information available on different vaccine formulations against CSFV is too diverse to allow the best vaccine candidate to be identified. The vaccine dosage, the number of doses, the route of administration and the time between vaccination and challenge all differ between studies. The virulence and the viral strain used for challenge, the dose and route of administration of the challenge virus and the criteria used to estimate the success of vaccination also vary. Establishment of common vaccination and challenge schedules, along with follow up criteria, will be necessary for objective comparison of vaccine types and selection of optimal vaccination strategies against CSFV.

CSFV mainly infects antigen presenting cells, such as endothelial cells, macrophages and DCs, and at the same time induces bystander apoptosis in surrounding B and T cells. Virulent CSFV strains have evolved in vivo to become more attenuated, thus avoiding death of the infected animal before the virus can spread to neighbouring animals. Mimicking viral infection by immunising with a DNA vaccine encoding the full-length recombinant CSFV E2 glycoprotein clearly induces T cell responses and primes for neutralising antibodies capable of conferring total protection against CSFV.

It is advisable to include other parameters, in addition to detection of humoral immune responses, when evaluating the efficacy of vaccines against CSFV. In most published CSFV vaccine studies, in particular those using classical live attenuated vaccines, detection of neutralising antibodies was the only parameter taken into account. There is now evidence that other arms of the immune system, such as T cell responses, are important in conferring protection against CSFV replication (Balmelli et al., 2005; Ganges et al., 2005; Suradhat et al., 2006).

Although techniques for analysis of T cell immune responses are not currently being used in most diagnostic laboratories, an effort should be made to develop new diagnostic tools based on measurement of cellular immunity. Characterisation of cytokine profiles induced after vaccination or infection with different CSFV strains by using ELISA, enzyme-linked immunosorbent spot (ELISPOT) or lymphoproliferation assays might become useful diagnostic tools in the future. One of the advantages of such techniques is that they may allow immunological status to be analysed at very short times after vaccination, even before antibody responses are induced (Suradhat et al., 2001, 2007)

In spite of the difficulties experienced in developing effective recombinant vaccines against CSFV, recent advances in this field offer promising possibilities for the future. The protective immunogens and the mechanisms of protection against CSFV have now been identified, which will allow the design of new and more effective marker vaccines.

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