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HUMORAL AND CELL IMMUNE MECHANISMS UNDER AFRICAN SWINE FEVER

(review)

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Abstract

The evidences for protective immunity against African swine fever are symptomless infection in local populations of *Phacochoerus africanus*, *Potamochoerus porcus*, *Hylochoerus* spp. In Africa and experimentally produced avirulent strains of African swine fever virus (ASFV), preventing death in pigs inoculated with its virulent homolog. Serum or colostrum from convalescent animals can delay clinical symptoms, decline viremia and increase the survivors' rate (R.C. Knudsen et al., 1987; D.H. Schlafer et al., 1984; D.V. Onisk et al., 1994). Humoral immune response is most probably due not to ASFV neutralization but the complement-mediated cytolysis (CMC) and antibody-mediated cell cytotoxicity (AMCC) (V.V. Makarov, 2013). Cell-mediated defense mechanisms, lyses of infected cells by N-killers and cytotoxic T-lymphocytes (CTL) lead to chronic or symptomless infection (S.G. Norley et al., 1983, 1984). An in vivo depletion of CD8+ T-lymphocytes by monoclonal antibodies was shown to stop protective immunity in pigs pre-immunized with an attenuated ASFV strain under next inoculation with the virulent homolog (C.A.L. Oura et al., 2005). Induction and defector activity of AMCC and CTL depend on the dose and biological features of ASFV strain (A.D. Sereda, 2010). High AMCC since day 3 and activity of cell-mediated mechanisms of immunity on day 6 after the ASFV inoculation provide a rapid decrease of the virus titers in blood, and, consequently, the absence of clinical symptoms (A.D. Sereda et al., 1992). Humoral and cell-dependent defense mechanisms act synergistically that must be taken into consideration when developing candidate recombinant vaccine against ASFV. ASFV candidate proteins involved in antigenic properties which can induce protective mechanisms against ASF are nominated. In pigs immunization with p54 and p30-32 combination caused a delay in appearance of ASF clinical symptoms after the inoculation with virulent isolate (P. Gymez-Puertas et al., 1998). CD2v (or GP 110-140) is a membrane protein of ASFV determining hemadsorption and identified as a serotype specific one (J.M. Rodriguez et al., 1993; A.D. Sereda et al., 1992; A. Malogolovkin et al., 2014). Therefore it should be considered crucial when constructing experimental protective facilities (H.H. Takamatsu et al., 2013). Thus, the investigations conducted to date show both humoral and cell effector mechanisms to be involved in defense against ASF.

Keywords: African swine fever, protective immunity, antibodies, cytotoxic T-lymphocytes, N-killers.

African swine fever (ASF) is a contagious septic viral disease of pigs, characterized by fever, toxicity signs, hemorrhagic diathesis and high mortality. In acute, the most common form of infection, 100 % animals die within 5-10 days after the clinical signs occur. However, a number of ASF virus isolates (African swine fever virus, ASFV; *Asfarviridae*) highly pathogenic for domestic pigs do not cause mortality in giant forest hogs (*Hylochoerus* spp.), red river hogs (*Potamochoerus porcus* JE Gray, 1821), bushpigs (*Potamochoerus larvatus* F. Cuvier, 1822) or common warthogs (*Phacochoerus africanus* Gmelin, 1788) in Africa. A variety of virus isolates, strains, variants and clones, which do not cause death in domestic pigs, and are able to generate protection against subsequent infection with homologous though not heterologous virulent isolates have been

isolated in nature and in vitro by attenuation in cell cultures or directional changes of the viral genome [1-4].

Russian scientists have experimentally substantiated the classification of ASF virus isolates by their seroimmunotypes, and obtained live-culture vaccines from attenuated strains for temporary protection of pigs against ASF seroimmunotypes I-IV [5-7]. All this gives evidence of the development of immune protection against ASFV in animals.

Investigations in the ASF immunology includ the functional status of the immune system organs and cells at various forms of the disease, the immunomodulatory effect of the virus on macroorganism and immune competent cells, serologic and immune plurality of the pathogen, search for protective proteins, etc. [5, 7].

The objective of the present review is to discuss the role of humoral and cell-mediated immune mechanisms in the development of protection against African swine fever.

Humoral immunity. Antibodies are considered as a necessary component of protective immunity in ASF. Administration of serum or colostrum from convalescent pigs to intact ones may delay the disease clinical manifestation, reduce viremia and increase the percentage of survivors in case of subsequent infection with a virulent isolate of ASFV [8, 9]. In particular, passive transfer of convalescent serum has protected 85 % of the pigs from subsequent infection with the homologous isolate E75, with a 100 % mortality rate in the control animals injected with immunoglobulins from an intact animal [10]. However, the mechanisms underlying humoral immunity in ASF remain debatable for a long time.

Virus-neutralising antibodies. Antibodies neutralising ASFV were first discovered in vitro by J. Parker and W. Plowright in 1968 [11]. Various experiments conducted by different research groups in the 1980s allowed to conclude that the virus did not induce such antibodies [12, 13]. However, since the 1990s, more and more support was given to the hypothesis about the importance of neutralizing antibodies in the protection against ASF, which was reflected in a comprehensive review by J.M. Escribano et al. [14].

They demonstrated that serum from a convalescent pig inoculated with the attenuated strain E75CV1-4 was able to neutralize 86-97 % infectivity of virulent isolates in the Vero cells cultures and porcine macrophages, and not only of the original isolate E75, but also of E70, Lisbon 60, Malawi Lil 20/1, and E75CV/V3 (a low-passage E75 virus). Unexpectedly, such immune blood serum was unable to neutralize the high passage ASFV (Lisbon 60, Haiti, Dominican Republic I, Dominican Republic II, Brazil II). Similar results were obtained with monoclonal antibodies to P72. The authors suggested that the virus adaptation during passaging in cell culture led to the loss of specific determinants involved in its neutralization. However, later it was reported that the observed differences were not related to the antigenic structure of the virus but caused by changes in the phospholipid composition of cell membranes [14-16].

Investigation of blood serum from convalescent animals showed that the ASFV p72, p30 and P54 proteins had the highest potential of antigenicity and immunogenicity in infection. Swine serum against a recombinant protein obtained by expression of a construct comprising genes p72 (capsid protein), p30 and p54 (inner envelope proteins) neutralized 70 % infectivity of ASFV. Anti-p72 and anti-p54 antibodies inhibited the attachment of virions to cells, while anti-p30 antibodies suppressed the penetration of the virus into the cells [17-19]. Interestingly, the antiserum against the recombinant envelope attachment protein p12 did not reduce the infectivity of ASFV in vitro [16]. Immunization of

pigs with a combination of p54 and p30-32 viral proteins postponed the onset of clinical manifestation of the disease in animals infected with virulent isolate [20-22].

Russian researchers have not validated experimentally a neutralizing activity against ASFV. By titration of the ASFV F-32 strain after its incubation with antibodies from convalescent animals and a complement no neutralizing effect was showed, including that of a complement-dependent virolysis [23]. Electron microscopy data show that the virion—antibody immune complex freely enters sensitive cells. According to V.V. Makarov [24], the specificity of macrophages, the target cells for ASFV, is primarily due to phagocytic activity, so initially no receptor mechanisms are needed for virus interaction with a cell. Furthermore, the ASFV size makes it the subject to phagocytosis competence, and opsonization of the virions as a result of the formation of immune complexes on their surfaces inevitably promotes their penetration and disintegration via opsonin-mediated activation of phagocytosis [23, 24]. In line with this findings is the data on pigs' experimental immunization with a pool of virus-specific glycoproteins purified by affinity chromatography. Despite the presence of high titers of serum antibodies to a wide variety of the ASFV glycoproteins, confirmed by immunoblotting, which were actually comparable to those in pigs recovered after ASF, the animals infected with a homologous virulent strain died 2-3 days earlier than non-immunized controls (A.D. Sereda, unpublished data)

Antibody-dependent cellular citotoxicity. Some phenomena, especially those observed in vivo, which had been previously explained by virus neutralization, possessed a different interpretation when the antibody-mediated cytolysis of ASFV-infected monocytes and macrophages was observed in cell-dependant citolysis CDC and ADCC reactions [25-27]. ADCC was recorded on day 3-6 after the pigs inoculation with the attenuated strain FK-135 in a dose of 10^8 HAU_{50} or a moderately virulent strain FNG in a dose of 10^3 TCD_{50}. As antibodies to 14, 30, 38, 69 and 95 kDa polypeptides were identified in blood serum of animals by radioimmunoprecipitation within the same time, it may be suggested that they (or some of them) do mediate the observed cytotoxicity [27]. N.G. Shubina et al. [28] reported that immunisation of pigs with purified p14, p25, p28, p31, p38 and p73 proteins induced lysis in the cells infected with ASFV via CDC and ADCC mechanisms.

Cell-mediated immunity. There is evidence indicating an important role of cell-mediated immune mechanisms for pig survival under ASFV infection, in particular, modification of N-killer activity and induction of cytotoxic T-lymphocytes (CTL) have been shown [29, 30].

Natural killer-cells. The role of N-killers (NK) in the protection against ASF has been shown in studies using pigs inoculated with the avirulent isolate NHV, when two forms of infection, asymptomatic and chronic, were observed. In acute and subacute forms of ASF, the activity of N-killers decreased on days 3 to 6 after the infection and further approached to normal level in the survivors. No changes were observed in N-killer activity both in pigs with chronic ASF and intact animals, though viremia and fever as well as a high titer of virus-specific antibodies were recorded 14 days after infection. In asymptomatic infection the viremia was rarely seen in the late stages of infection, the concentration of specific anti-ASFV antibodies was relatively low, though the activity of N-killers became very high. Such animals were clinically asymptomatic after infection with a virulent strain L60 [31). The results obtained suggest an important role of N-killers in resistance of pigs to ASFV infection.

Cytotoxic T-lymphocytes. The role of CTL in providing virus-specific protection in the early stages after ASFV infection was first demonstrated in the

mid-1980s [30, 32, 33]. It was shown that only the purified CD8+ T-cells, but not purified CD4+ lymphocytes possessed killer activity, and that function was blocked by anti-CD8+ antibodies.

The ASFV-specific activity of CTL was investigated in inbred minipigs with different types of the swine major histocompatibility complex or SLA (Swine leukocyte antigens) [34]. The animals were experimentally inoculated with the avirulent ASFV isolate NHV. Secondary CTL were activated by in vitro re-stimulation of effector T-lymphocytes in blood leukocyte cell culture infected with low doses of a homologous virus isolate at infection multiplicity of 0.1 for 48 to 72 hours. The CTL activity was found to be limited by the presence and functional activity of SLA class I: first, inhibition of the target cell antigens by monoclonal antibodies against SLA Class I resulted in a significant reduction in CTL activity; second, the preferred lysis of ASFV-infected target cells with SLA Class I was observed; thirdly, the destruction of CD8+ effector cells by specific monoclonal antibodies and complement led to a decrease in CTL activity. Lysis of macrophages by cytotoxic T lymphocytes was higher if macrophages were infected with a homologous virulent isolate L60 compared to the infection with heterologous isolates DR-II and Tengani [34], being the evidence of the CTL immune specificity.

The crucial role of virus-specific CTLs in the defense against ASF was confirmed by the protective immunity abrogation under the in vivo depletion of CD8+ T-lymphocyte pool by monoclonal antibodies [35]. In the pigs, inoculated with the attenuated strain OUR/T88/3 and administered with monoclonal antibodies against CD8+ T-lymphocytes (for 5-6 days in a month after immunization), the protection against subsequent infection with a virulent strain OUR/T88/1 were lost, and viremia and fever developed.

Note, the primary CTL detection methodologically depends on the dose and biological properties of the ASFV strains. This fact was clearly shown by A.D. Sereda [36] under the limitation on SLA Class I which was achieved when A-cells culture of peripheral blood leukocytes from a pig before virus inoculation (day 0) were used as target cells, and the autologous peripheral blood lymphocytes taken on days 1-12 after ASFV inoculation were the effector cells [37]. On days 6-8 in pigs infected with attenuated strain FK-135, the specific cytolysis of A-cells in the presence of effector cells reached 20 % at a dose of $10^{8.5}$ HAU_{50} and decreased to 5 % at 10^{6} HAU_{50} and 0 % at 10^{3} HAU_{50} doses. However, the cell-mediated cytolysis of target cells infected with strains F-32 and FK-135 was not induced in pigs inoculated with virulent strain F-32 in the above doses [36]. This fact apparently indicates that in the strain F-32 the immunodominant epitopes of a target protein are somehow masked. The problem of antigen presentation seems to be manifested not only at the effector response level, but also in the course of recognition by T helpers. In splenocytes of a pig, which was inoculated with strain FK-135, the interleukin-2 (IL-2) production in the presence of A-cells infected with the strain FK-135 was the same as mitogen-induced production. In the presence of A-cells infected with a moderately virulent strain FNG, the IL-2 production was 2.5 times lower, while with the strain O-32 it was 6-7 times lower. Note, in the presence of autologous A-cells infected with a immunotypically heterologous strain Mozambique-78 no IL-2 production was found in splenocytes of a pig inoculated with the strain FK-135.

The synergy of protective immune mechanisms. Based on the duration of the incubation period and the timing of animal deaths from ASF, it may be suggested that the disease outcome is determined by the dynamics of the humoral and cell-mediated defense mechanisms in the first few days after infection. This hypothesis was experimentally confirmed in the models using homologous ASFV

strains of unequal virulence, i.e. F-32 (virulent), FK-135 (avirulent) and FNG (moderately virulent) [36]. In pigs inoculated with FK-135 at a dose of 10^8 HAU_{50}, the CTL-mediated and NK-dependent cytolysis of the autologous A-cells infected with strain FK-135 and sampled on day 0, peaked on day 6, whereas in pigs infected with strain F-32 at a dose of 10^3 HAU_{50} or FNG at 10^3 TCD_{50} dose, the virus-specific cell-mediated cytolysis (CMC) was not found. In ADCC, the antibodies were identified only starting from day 3 after the animals' inoculation with FK-135 and FNG. In animals infected with the strain F-32, the ADCC activity of antibodies was found only the day before death, i.e. on day 6. Thus, under infection with strain F-32, CMC and ADCC were not recorded for 3 to 6 days after inoculation, and these pigs died on day 8. Lack of CMC on day 6 after FNG inoculation, combined with the presence of ADCC starting from the day 3, resulted in the development of ASFV chronic infection. High titers of ADCC 3 days after the FK-135 inoculation and CMC on day 6 ensured rapid reduction of the virus concentration in blood on days 3-6, and, consequently, no clinical symptoms were noted.

The role of humoral and cell-mediated mechanisms in the protection against ASF was shown in studies of the cytotoxicity parameters in pigs inoculated with the attenuated strain FK-135 at 10^3 to $10^{8.5}$ HAU_{50}. Animals were divided into two groups based on ADCC and CMC values measured on day 3 and day 6, respectively. ADCC was 10 % to 20 % and CMC was 3 % to 10 % in the first group, and ADCC was 0 % to 10 % and CMC was about 0 % in the second group. After intramuscular challenge with a virulent strain F-32 at 10^3 HAU_{50}, the animals in first group remained clinically healthy on day 7, while the second group suffered from infection with fever for 2-3 days [38].

The cultures of swine peripheral blood leukocytes can be used for in vitro study of immunological processes, which take place in vivo over the specified time period. It is quite easy to divide the leukocyte culture into three components, i.e. glass-adherent target cells (macrophages, A-cells), non-adherent pelleted effector cells (CTLs + N-killers), and the culture medium with blood antibodies remaining in the supernatant. The final effects of antibody-mediated (CMC plus ADCC) and cell-mediated (CTLs plus N-killers) immune mechanisms in limiting reproduction of the ASFV strain F-32 were found out in the autologous model systems, when the cell cultures derived from peripheral blood leukocytes of the same pig sampled before and 6 days after the animal immunization with the attenuated ASFV strain FK-135 at $10^{7.5}$ HAU_{50} were used [39]. In case of lymphocytes and serum sampled after immunization, the ASFV accumulation in the cultures was reduced by 2.00-2.50 lg HAU_{50}/cm^3 compared to the control with lymphocytes and serum from an intact pig. In the presence of lymphocytes from an immunized animal and the serum from an intact one, ASFV accumulation decreased by 0.67-0.83 lg HAU_{50}/cm^3. Finally, in the presence of lymphocytes from an intact pig and antibodies from an immunized pig, it decreased by 1.34-1.83 lg HAU_{50}/cm^3. Therefore, on day 6, the antibody-mediated defense mechanisms were superior to cell-mediated ones in restricting ASFV reproduction [39]. Based on the results obtained, both mechanisms act synergistically, which is of key importance to be taken into account when developing recombinant protective agents against the ASFV. Besides, the effector mechanisms can possibly act against various epitopes in different virus-specific proteins.

The protective function of antibodies in ASF is due to the ADCC and CDC mechanisms. Assuming that pools of T- and B-lymphocytes induced by ASFV are accumulating at the same rate, the effect of B-lymphocytes should be

prompt as each such cell produces large amount of antibodies that can be used by N-killers to attack infected target cells. It has been experimentally confirmed that ADCC can be detected 3 days after infection, while primary CTLs are recorded on days 6-8. However, CTL induction must be considered as critical for effective control of viral replication [40, 41].

It appears that the virus-specific protection against ASF is provided via a set of proteins that induce the two types of immune response mechanisms, however, investigation of the structural and functional organization of ASFV proteins does not suggest a clear understanding which ones can be considered as relevant antigens. Based on the localization of virus-induced proteins in the virion envelope and the plasma membrane of infected cells, and taking into account the dynamics of antibodies to specific viral proteins, and the effect of pig immunization with various recombinant constructs, there are at least several candidate proteins, e.g. p30, p54 and CD2v [42, 43]. The latter determines haemadsorbing properties, promotes ASFV dissemination in the pig body, is expressed as a glycoprotein with a molecular weight of about 105 kDa, and possesses the immunomodulating properties [44-48]. We believe that the protein CD2v corresponds to a major glycoprotein with a molecular weight of 110-140 kDa (GP 110-140) found in the plasma membrane of infected bone marrow A-cells of pigs. Unlike other ASFV proteins, the glycoprotein GP 110-140 is serologically specific [49-51]. In view of the found out serological immune specificity in the protection against ASF, the crucial role of CD2v (GP 110-140) in the development of protective immunity appears to be very likely.

Thus, the studies available to date suggest the participation of humoral and cellular immune mechanisms in developing protection against African swine fever (ASF). Despite the experimental evidence for partial neutralization of the ASF virus (ASFV) in vitro, it should be recognized that the opposite point of view, according to which the development of the complex of antibodies with the virion leads to opsonized phagocytosis and, as a result, to the increased likelihood of monocyte/macrophage infection through a «Trojan horse» mechanism, seems to be more reasonable, especially in vivo. Most likely, the humoral component of immune system does not occur via neutralizing ASFV, but with the participation of complement-dependent cytolysis (CDC) and antibody-dependent cellular cytotoxicity (ADCC). Induction of CTL is also critically important for effective control of the viral replication. Activation of the cell-mediated defense mechanisms, lysis of infected cells by natural killers (N-killer cells) and cytotoxic T lymphocytes (CTL) ensure the survival of the pigs or asymptomatic infections. The induction of CTLs depends on the dose and biological properties of ASFV strains. The humoral and cell-mediated protective mechanisms act synergistically, which is of key importance to be taken into account when developing recombinant vaccines against ASF. CD2v (or GP 110-140) is a membrane protein of ASFV, which determines the haemadsorption and is serotype-specific. Therefore, it must be critical in designing protective anti-ASF agents.

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