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Original article

Pig Major Acute-Phase Protein and apolipoprotein A-I responses correlate with the clinical course of experimentally induced African Swine Fever and Aujeszky's disease

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Abstract – In the present work, we studied the acute phase protein response after experimental virus infection in pigs. The animals were experimentally infected with African Swine Fever (ASF) or Aujeszky's disease (AD) viruses. The clinical course of ASF infection correlated with increasingly high levels of pig Major Acute-phase Protein (pig-MAP) (mean value of 6 mg/mL on day 6 post infection (p.i.), from 6 to 9 times higher than day 0) and sharp apolipoprotein A-I (apo A-I) decrease (mean value of 0.5 mg/mL, from 4 to 10 times lower than day 0 on day 4 p.i.). AD-clinical signs appeared at day 3 p.i., both in vaccinated (moderate clinical signs) and non-vaccinated pigs (severe outcome within 48 h p.i.). Pig-MAP and apo A-I profiles also followed clinical signs (changing from 0.70 mg/mL to around 3 mg/mL and from around 3 mg/mL to 0.96 mg/mL, respectively in non-vaccinated animals), with minor changes in concentration in the vaccinated group. Haptoglobin levels significantly increased in ASF and AD infected animals (mean maximum values of 2.77 and 3.96 mg/mL, respectively). Minor differences for the C-Reactive Protein in the case of ASF were observed, whereas its concentration increased more than 7 times in AD-infection. The albumin level was not modified in either case. The correlation of clinical signs to our data suggests the potential use of pig-MAP and apo A-I in monitoring infections in swine.

pig-MAP / apo A-I / African Swine Fever / Aujeszky's disease / pig

1. INTRODUCTION

Acute phase response is a non-specific complex set of systemic reactions that occur as a consequence of infection, inflammation or trauma. This response is mediated by inflammation-related cytokines (mainly by interleukin-6 (IL-6), interleukin-1 (IL-1) and tumour necrosis factor alpha (TNF-α)), for which the liver

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is the principal target. This organ controls the plasma levels of acute phase proteins (APP) during the critical stages of inflammatory or infectious diseases. The concentration of these proteins increases or decreases within hours or days after the onset of these processes [3, 19, 20]. Studies have shown important variation in the APP response among species. APP were first characterised in humans [20], rodents [19, 22] and later in other animal species, such as pigs [13, 21]. Clinical use of APP determination has been proposed in veterinary medicine to determine the health status of farm animals in various production processes [14, 28, 39]. Increases in haptoglobin (Hp), C-reactive protein (CRP) and serum amyloid A in pigs with induced inflammation or bacterial infection have been reported [13, 18, 21]. In previous works carried out at our laboratory, a new 120 kDa plasma glycoprotein, which was denominated Pig Major Acute Phase protein (Pig-MAP), was identified as a relevant acute phase protein in animals under turpentine-induced inflammation [17, 21]. This protein was also referred to as PK-120 [26] or IHRP [36] being subsequently denominated ITIH4 (heavy chain 4 of the inter-alpha trypsin inhibitor family) [5]. Increases in the ITIH4 concentration under different physio-pathological situations have been reported in pigs [18, 21, 38], rats [10, 17], bovines [30], humans [29, 43] and mice [12]. In addition, we have previously reported that apolipoprotein A-I (apo A-I), the major protein component of high density lipoprotein (HDL), shows prominent decreases during experimental bacterial infection and acute inflammation [7, 25]. Decreases in apo A-I concentration during acute phase processes have been described in other species [27, 43], suggesting that it could be involved in the modulation of inflammation [1, 6]. Slight decreases in albumin and transferrin concentrations were also observed in pigs after turpentine induced inflammation [21].

Growing interest has been focussed on APP response given the fact that it may differ within the same species depending on the inducing agent (i.e. bacteria, virus, and trauma). It has often been suggested that in humans, viral infections caused a weaker response compared to that of acute bacterial infections [41]. However, not much is known about APP response elicited by viruses in pigs [2, 11, 35, 38]. The aim of this work was to give a general view of the APP response elicited during two relevant viral infections that have been extensively studied in swine: African swine fever (ASF) and Aujeszky's disease (AD). ASF is one of the most dreaded epidemic diseases of pigs. It is caused by a large, enveloped DNA virus that induces an acute haemorrhagic disease characterised by severe immune cell depletion and apoptosis induction in target and immune defence cells [33]. The clinical outcome of the disease ranges from lethal to moderately virulent or non-virulent according to the virus strain [4, 34]. A generalised infection rapidly follows via the bloodstream and is associated with a high fever (up to 42 °C) and acute death of the animal. AD is caused by an *alpha-herpesvirus* that infects the central nervous system and other organs, such as the respiratory tract. This virus can become latent in the trigeminal ganglia in pigs [9, 32]. It can infect virtually all mammals except humans, but swine are the natural hosts and the only reservoirs to become latently infected. AD causes high mortality in young pigs and a respiratory illness in older pigs. Vaccines are available and eradication plans have been implemented.

We report here the time course of the APP response in terms of serum concentration of pig-MAP, Hp and CRP, as well as apo A-I and albumin in swine undergoing experimental ASF and AD. Moreover, clinical signs of both diseases have been evaluated together with the APP response. Furthermore, an experimental

model consisting of pigs exposed to AD viral infection that were previously vaccinated against AD virus was included in the study.

2. MATERIALS AND METHODS

2.1. Animals and experimental acute phase models

2.1.1. ASF virus (ASFV) infection

Seven Landrace and Large White pigs (3 months old) were obtained from a farrow-to-finish farm, certified as having a high health status. Five were inoculated intramuscularly with 5×10^2 tissue culture infective doses (TCID50) of the high virulent ASF virus isolate E75, representing a challenge of 50 to 500 lethal doses LD_{100}. This is the dose commonly used for a challenge experiment to obtain an infection pattern in experimental animals similar to that of a highly virulent virus isolate in the field, with infection and death of more than 95% of the animals. Two animals were left uninfected as controls. Clinical signs of ASF (fever, anorexia, lethargy, shivering, cyanosis and recumbency) were monitored daily until day 6 post infection (p.i.). Viremia titer determination was performed in swine peripheral blood mononuclear cell (PBMC) cultures by cytopathic effect. Titres were calculated and expressed as 50% tissue culture infectious doses (TCID50) per mL as previously described [15]. Blood samples were collected prior to virus inoculation and on days 3 to 6 p.i.

2.1.2. AD virus (ADV) infection

Fifteen pigs born in a herd free of Aujeszky's disease were used in this part of the study. The sows were not vaccinated against AD in this herd. All the animals were ADV-seronegative. At 18 weeks of age (average live weight = 80.5 kg, SD = 8.8) the pigs were challenged three times 12 h apart, with a reference strain 75V19 of ADV, as described by Vannier et al. [40]. Eight of these pigs (AD I animals) had been previously vaccinated at the age of 10 weeks. The vaccine was a non-commercial live experimental vaccine of the Bartha strain, and was intramuscularly injected in a dose of 10^5 TCID50/mL (2 mL) after dilution in an Oil/Water 25% adjuvant. The other seven pigs (AD II animals) had not been vaccinated. Each pig was submitted daily to a detailed clinical examination of the parameters, including alertness, coughing, sneezing, respiratory distress, laboured breathing and the recording of rectal temperature. The pigs were weighed individually each week. Nasal swabs were taken daily, from day 3 p.i. to day 10 p.i. and again on days 13 and 14 p.i., to determine viral excretion, as previously described [40]. Blood samples were obtained before the injection (day 0) and on days 3, 5, 7, 14 and 21 days p.i. White blood cells were determined by conventional haematological counts.

In all cases of animal handling, stressful manipulation was kept to a minimum. All the experiments were performed with animals in experimental level biosecurity facilities, and were approved by the Animal Experimentation Committees or Ethical Committees of the institutes concerned. All experiments complied with the relevant EU guidelines on animal experimentation.

2.2. Protein isolation, antisera generation and immunochemical analysis

Pig-MAP, Hp, CRP, apo A-I and albumin were isolated from pig sera, according to previously reported protocols [7, 17, 21], and subsequently used either as primary standards or as antigens to generate the corresponding specific polyclonal antisera by subcutaneous inoculation in

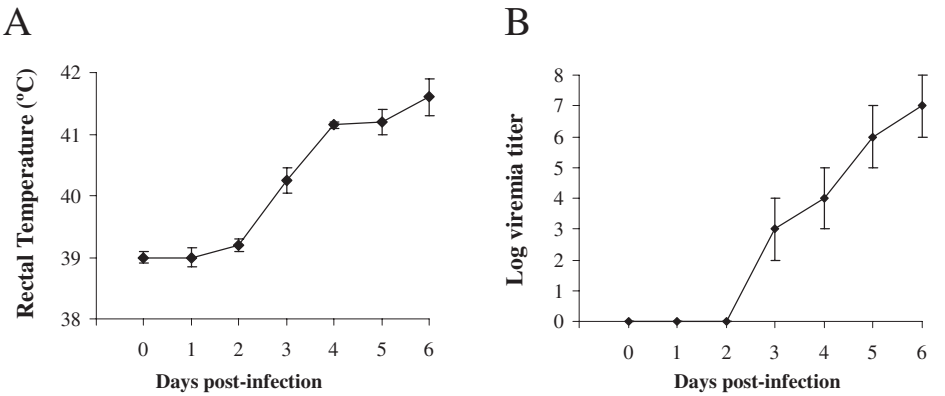

Figure 1. Time course of clinical signs of animals suffering from experimental ASF. Data are expressed as mean values ($n = 5$) of the following: (**A**), fever (rectal temperature, °C); and (**B**), viremia titer. Vertical bars show the standard deviation (SD).

rabbits [7, 17, 21]. The concentrations of the individual proteins in the sera were determined by radial immunodiffusion [23] in 1% agarose gel containing specific antiserum, as previously reported [7, 17, 21]. A pig serum, previously calibrated with the isolated proteins was used as the standard [7, 17, 21]. Differences in the protein patterns, between normal and sera from ASFV infected pig sera were analysed by crossed immunoelectrophoresis and peaks were characterised as previously described, using sera from pigs with experimentally induced acute inflammation [21].

2.3. Statistical analysis

An ANOVA test followed by a Tukey post hoc test was used for drawing comparisons between APP concentration levels, using the statistical computer program SPSS 12.0. For both infections, the significance of the data was evaluated on each experimental day p.i. and compared to pre-infection levels (day = 0). For the pigs infected with ADV, the differences between vaccinated (AD I) and non-vaccinated (AD II) animals were also compared on each experimental day.

3. RESULTS

3.1. ASFV infection

Clinical signs of ASFV infection, i.e. fever (Fig. 1A, rectal temperature greater than 40 °C), anorexia, lethargy, shivering, cyanosis and recumbency, appeared 3–4 days p.i. and progressed until death (days 5-6 p.i.). Viremia titers increased over time (Fig. 1B) and peaked at day 5 in one animal and at day 6 in four animals, coinciding with the day on which the pigs died. Anorexia and fever was found in all animals, lethargy, recumbency and cutaneous erythema in 20% of them. Briefly, necropsy revealed a haemorrhagic splenomegaly and haemorrhagic lymph nodes. Only red pulp was evident in the spleen, while white pulp was barely seen. Submandibular, mediastinal, mesenteric, gastrohepatic and renal lymph nodes, among others, were enlarged and showed white necrotic and hemorrhagic areas. There was marked macrophage and lymphocyte apoptosis in the remaining white pulp in the spleen and lymph nodes.

Figure 2 shows the representative crossed immunoelectrophoresis pattern of

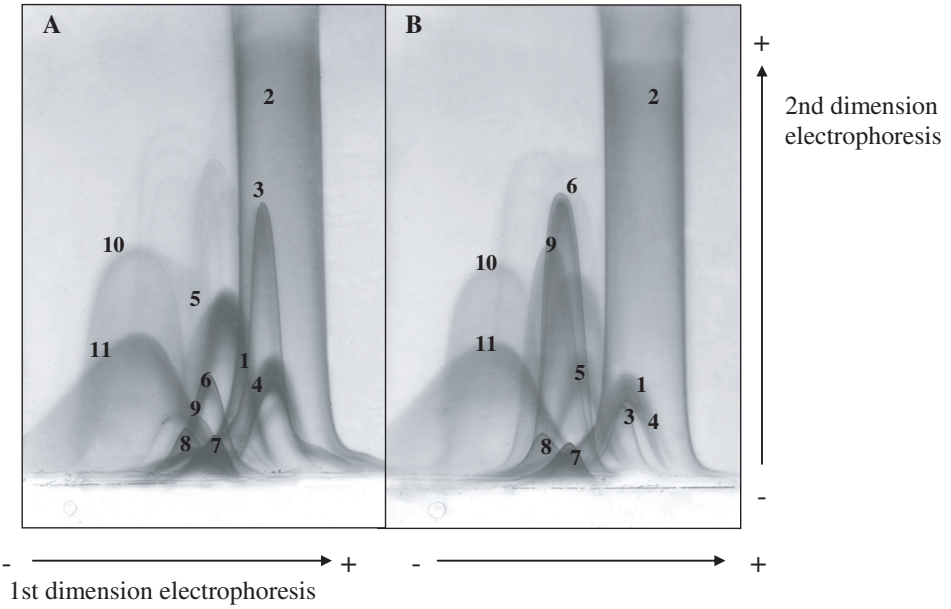

Figure 2. Crossed immunoelectrophoresis of serum from the same pig: (**A**), at day 0; and (**B**), 5 days after ASFV inoculation. The numbered peaks correspond to the following: (1) α1-antitrypsin, (2) albumin, (3) apo A-I, (4) α1-acid glycoprotein, (5) fetuin, (6) haptoglobin (7 and 8) α2-macroglobulin, (9) pig-MAP, (10) transferrin and (11) IgG.

proteins in serum from the same pig collected before (Fig. 2A, normal pattern) and after 5 days of ASFV infection (Fig. 2B, acute-phase pattern). The Hp and pig-MAP peaks (6 and 9, respectively) greatly increased in the acute phase serum while peak 3 (apo A-I) decreased. There was also a slight decrease in peak 5 (fetuin).

Time-course analysis of APP concentrations in sera from ASF pigs revealed significant variations (Fig. 3). Pig-MAP, Hp and apo A-I response was coincident with the onset of fever (Fig. 2A) and other clinical signs. The pig-MAP concentration began to increase on day 3 p.i. reaching maximum values on day 6 p.i. of around 6 mg/mL (range: 5.80–6.27 mg/mL; $P \leqslant 0.001$ referred to day 0, 6 to 9 times higher than initial values). The evolution of the pig-MAP followed the pattern observed for the viremia levels (Fig. 2B). The Hp maximum level was reached before that of the pig-MAP, 4-5 days p.i. (range: 2.05–3.16 mg/mL; $P \leqslant 0.001$ referred to day 0). The CRP concentration was not highly modified during the first 5 days, but an increase in its concentration was observed on day 6 p.i., reaching mean values of 0.06 ± 0.02 mg/mL. A significant decrease in the concentration of apo A-I was observed from day 3 p.i. Minimum concentrations (a quarter to a tenth of the initial values) were reached on days 4-5 p.i. (range: 0.21–0.68 mg/mL; $P \leqslant 0.001$ referred to day 0). These values were maintained until the end of the trial. In the case of albumin, only a slight and no significant decrease on concentration was observed on days 5 and 6 p.i. No significant variations in any of these parameters were found in the serum from two uninfected control pigs. APP mean values on day 3 p.i. were 0.88, 0.87, 0.03, 2.33 and 17.89 mg/mL for pig-MAP, Hp,

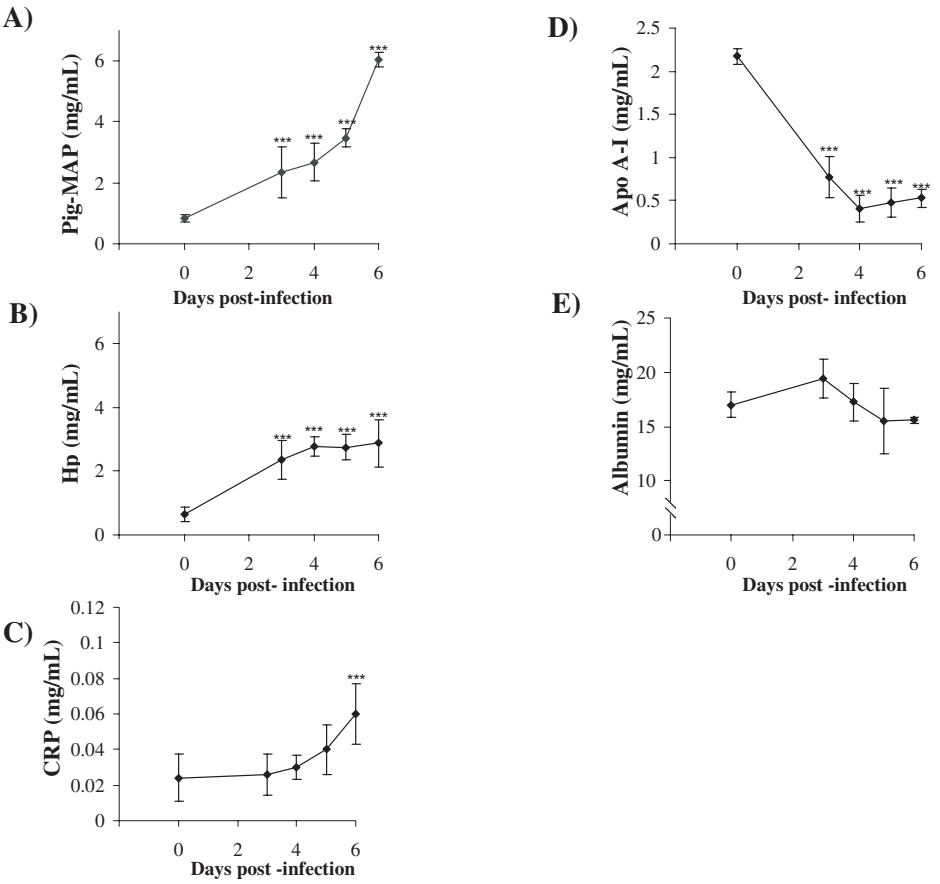

Figure 3. Time course of mean concentration of pig-MAP (**A**), Hp (**B**), CRP (**C**), apo A-I (**D**) and albumin (**E**) response in sera from pigs ($n = 5$) before (day 0) and at the indicated days after ASFV infection. The concentration of each protein was determined as described in Materials and Methods. Abscissas: days after the infection. Ordinates: Protein concentration, in mg/mL. * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$, statistical differences referred to day 0 using ANOVA followed by the Tukey post hoc test. Vertical bars show SD.

CRP, apo A-I and albumin concentrations, respectively, without modification in the following experimental days.

3.2. ADV infection

Clinical signs of AD including fever, anorexia, coughing, sneezing, weight loss and dyspnoea appeared from the first day of infection. All ADV infected animals displayed fever from 48 h p.i. (Fig. 4A). However, there were important differences between the two groups: AD I pigs showed clinical signs of moderate severity when compared with AD II pigs in terms of liveweight and fever. Their rectal temperature did not exceed 40.5 °C, returning to normal values after day 5 p.i. In contrast, AD II pigs developed severe AD

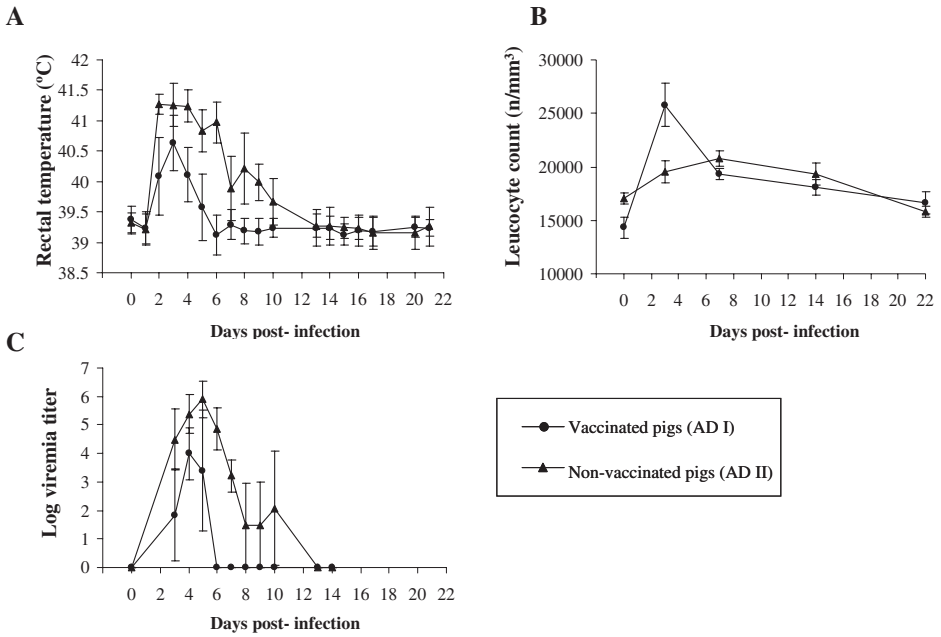

Figure 4. Time course of clinical signs of pigs suffering from experimental AD. Data expressed as mean values per group of vaccinated pigs (● symbol, n = 8, AD I animals) and non-vaccinated pigs (▲ symbol, n = 7, AD II animals). (**A**), fever (rectal temperature, °C); (**B**), leucocyte counts (n/mm^3); and (**C**), viremia titer. Vertical bars show the standard deviation (SD).

Table I. Body weight in different groups infected with Aujeszky's disease virus.

	Day -7	Day 0	Day +7	Day +14	Day +21
Non vaccinated pigs (AD-II)					
BW	72.1	79.1	72.4	81.4	89.1
SD	6.8	6.7	6.1	6.4	7.2
Vaccinated pigs (AD-I)					
BW	72.1	80.4	83.1	91.2	99.3
SD	9.3	10.5	12.0	12.7	13.7

BD: Body weight, kg; SD: Standard deviation.

symptoms (fever around 41 °C, maintained from 48 h until day 10 p.i.). On day 0, AD I and AD II animals had the same body weight. During the first week p.i., AD II animals seriously lost weight (from 79.1 to 72.4 kg) whereas those from the AD I group showed some body weight increase (from 80.4 to 83.1 kg). Although, these differences were maintained until day 21 p.i. (Tab. I), in the following days all animals exhibited body weight increase. The white blood cell count increased early after infection in the AD I group whereas the AD II group exhibited a lower and delayed response (Fig. 4B). All the AD II pigs excreted the virus at least until day 7 p.i. (Fig. 4C). The vaccinated animals showed reduced viral excretion and only

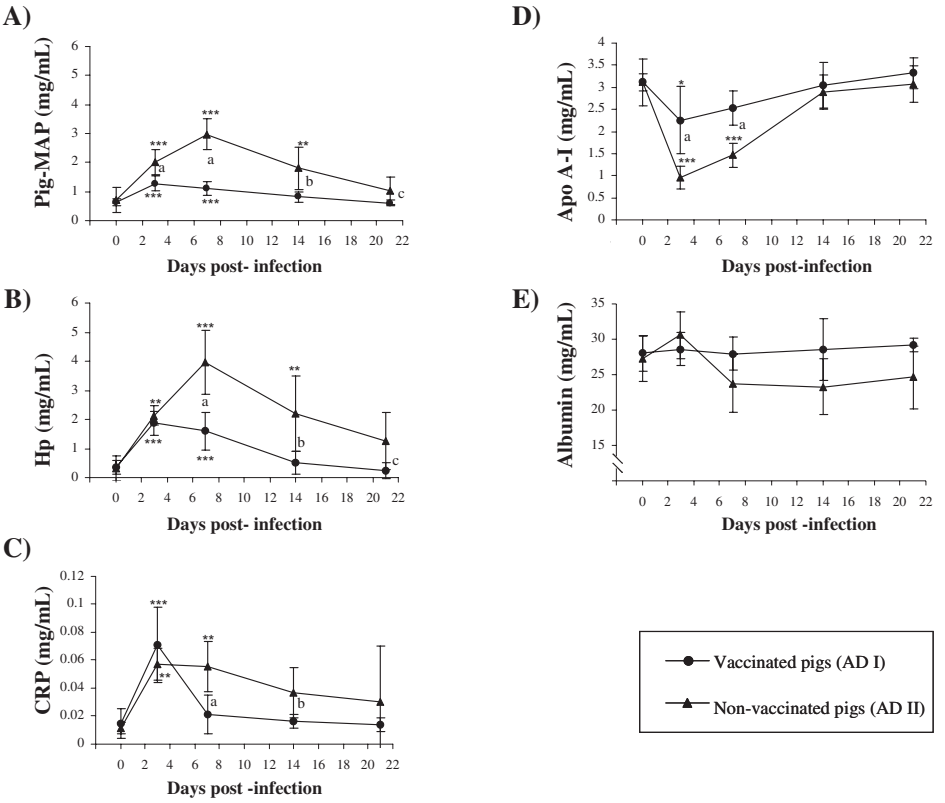

Figure 5. Time course of mean concentration of pig-MAP (**A**), Hp (**B**), CRP (**C**), apo A-I (**D**) and albumin (**E**) response in sera from pigs: before (day 0) and after ADV infection. Pigs previously vaccinated with an experimental ADV vaccine (● symbol, AD I animals), and non-vaccinated pigs (▲ symbol, AD II animals). The concentration of each protein was determined as described in Materials and Methods. * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$, statistical differences referred to day 0. Statistical differences between AD I and AD II animals were identified with letters: c, $P \leqslant 0.05$; b, $P \leqslant 0.01$; a, $P \leqslant 0.001$. Statistical analysis was carried out using ANOVA followed by the Tukey post hoc. Vertical bars show SD.

5 pigs excreted the virus on day 3 p.i. and none beyond day 5 p.i.

The time course of APP levels is shown in Figure 5. In non-vaccinated animals, a significant increase in pig-MAP concentration was observed from day 3 to 14 p.i., with respect to the serum concentrations before the infection (0.72 ± 0.44 mg/mL). The concentration of this protein on day 3 p.i. was 2.02 ± 0.44 mg/mL ($P \leqslant 0.001$ referred to day 0) and the maximum values were reached on day 7 p.i. (2.98 ± 0.54 mg/mL, $P \leqslant 0.001$ referred to day 0). Normal values were recovered after 21 days p.i. The temporal evolution of Hp concentration was similar to that of the pig-MAP. Nevertheless, Hp was strongly induced by ADV, thus the initial values (day 0) ranged between 0.02 and 1.19 mg/mL, exhibiting the highest values on day 7 p.i. (3.96 ± 1.10 mg/mL, $P \leqslant 0.001$ referred to day 0), when some

animals showed an increase of more than 10 times the basal concentration. CRP reached maximum levels on 3 days p.i., showing an increase of 7 to 11 times the initial values (0.057 ± 0.011 mg/mL, $P \leqslant 0.01$ referred to day 0), and then decreased to basal values. Pre-infection levels of apo A-I were 3.11 ± 0.19 mg/mL (range: 2.90–3.38 mg/mL). The concentration decreased sharply and on day 3 p.i. it showed minimum values of about a third of the initial values (range: 0.72–1.34 mg/mL; $P \leqslant 0.001$ referred to day 0), with an average concentration of 0.96 ± 0.27 mg/mL. The concentration began to increase slightly on day 7 p.i. (1.46 ± 0.27 mg/mL, $P \leqslant .001$ referred to day 0) and then returned to basal values on day 21 p.i. For albumin, only a slight decrease was found on day 7 p.i.

As with the clinical signs, there were significant differences in the serum APP concentrations between vaccinated and non-vaccinated pigs. The variation in the concentration of these proteins was lower in all vaccinated pigs (Fig. 5). In this group, the pig-MAP concentration peaked on day 3 p.i. (around 1.30 mg/mL) and thereafter decreased to the initial values. Significant differences in concentrations were observed between AD I and AD II groups on day 3 p.i. through to day 21 p.i. Hp levels showed a higher increase in both groups, and differences between AD I and AD II animals were only observed after day 7 p.i. In the case of CRP, there was a similar increase on day 3 p.i. in both groups but whereas the concentration levels remained high for non-vaccinated animals they returned to normal on day 7 p.i. in the case of vaccinated pigs. The vaccinated pigs showed a slight decrease in apo A-I concentration less than 2 times the normal values. The concentration of this protein then increased to reach basal levels on day 14 p.i. Differences between groups were observed on day 3 and 7 p.i. The albumin concentration was not modified in AD I pigs.

4. DISCUSSION

Inflammation caused by infections, chemical inflammatory agents and a variety of pathological conditions results in changes in the concentration of APP in serum. Research interest has been focussed on the study of pig APP response in experimental acute inflammation and bacterial infections, as well as in farm animal manipulations [7, 18, 21, 28, 31, 39, 42]. Although there have been studies about virus infections in pigs [2, 11, 35, 38], little is known about the APP response elicited by these pathogens.

With the aim of analysing the APP response in viral infections and establishing whether this response correlated with the clinical course of the disease, an overall picture was obtained of APP concentrations in two representative viral diseases in pigs, ASF and AD. These diseases follow different clinical courses and constitute appropriate model diseases for the present study. ASF is one of the most acute and severe diseases of pigs and there is no vaccine available. This viral infection has been the subject of a large number of studies related to different aspects of infection: pathogenesis, target cells, cytokine regulation [16, 37] and apoptosis of infected cells [8, 33]. The clinical course of ASF, the evolution of fever and the viremia titer profiles were very similar in animals inoculated with the same isolate and doses of the virus, as has been reported in the literature [4, 33, 34] but clinical signs are not sufficient to perform a differential diagnosis in the field. With respect to the serum markers reported here, the major changes in APP response were observed after 4 days p.i. for apo A-I and Hp, and 6 days p.i. for pig-MAP. Remarkably, pig-MAP sera levels in ASF animals were much higher than

in AD animals or in levels reported for pigs affected by PMWS [38]. Hp induction followed a different profile, showing maximum values at 4 days p.i. with no further increases. These maximum values were comparable to those of AD infection. The response of CRP, a well known increased APP, was quite different in the case of ASF. The CRP concentration was not highly modified during the first 5 days, but an increase in concentration was observed at day 6 p.i. In human medicine it is widely accepted that viral infections cause minor increases in CRP concentration compared to that of acute bacterial infections [41]. This could explain the low response of this protein in this viral infection. Another remarkable fact was the very low concentration reached by apo A-I (lower than 0.30 mg/mL in some animals), the lowest values reported for this protein in pigs [7, 25]. In conclusion, the apo A-I, Hp and pig-MAP profiles followed the clinical course of ASF. In this sense, the serum concentrations of these proteins were maintained at minimum and maximum levels, and did not return to the basal values since viremia increased and symptoms severed the clinical course leading to the death of the animals.

AD causes very high mortality in pigs younger than 4 weeks, but in older pigs it is less aggressive and lethal compared to virulent ASF. It has been reported that cell-mediated cytotoxic activity seems to play a major role in the fight against the virus [9, 24] allowing recovery of infected animals. Thus, the development of vaccines that can favour this immune mechanism has become the best tool to combat the disease [40, 44]. The role of innate immunity and that of APP in the first line of host defence against the viral infection may also contribute to the outcome of the disease. The low incidence of fatality and less acute clinical course of AD enabled a prolonged observation of the APP response. Apo A-I and CRP greater variations were reached earlier than pig-MAP and Hp (on day 3 and day 7 p.i., respectively). In the case of AD, the less pronounced changes in pig-MAP and apo A-I were in concordance with a moderate outcome of the disease. In contrast, Hp reached higher values in AD (around 4 mg/mL) than in ASF infection (around 3 mg/mL). Finally, although albumin concentration decreases have been reported during acute phase response [21], only minor variations were detected here in both diseases.

APP response was also analysed in animals previously vaccinated with a live experimental vaccine (AD I animals). They showed an early increase in the number of leucocytes following ADV contact and resulted in recovery after showing mild clinical signs. It is an optimal model to study the APP response, since those animals were subjected to the same experimental daily blood sampling routine as AD II animals but exhibited none or only mild clinical signs. As expected, APP showed a less pronounced response in the AD I group, achieving normal values in a shorter period of time. All proteins exhibited statistically significant differences between AD I and AD II animals. However, pig-MAP and apo A-I responses closely follow the course of the disease from day 3 p.i., whereas for the other APP studied these differences were not observed until day 7 p.i. It is interesting to point out that CRP values on day 3 p.i. were similar in both groups, although significant differences appeared in subsequent days p.i.

In conclusion, those APP quite clearly reflect the time course of the disease in ASF and AD virus infections. They also discriminate between vaccinated and unvaccinated animals in the AD infected groups, especially in the case of pig-MAP and apo A-I, which exhibited differences between these groups earlier than Hp and CRP. In the ASF infected animals, the maintained marked alterations of APP are witnesses of the incapacity of the organism

to overcome the infectious process. Moreover, our data suggest that APP are useful indicators of the clinical course of these virus infections and could constitute complementary tools for evaluating how quickly a vaccine could provide effective protection. More studies are needed to achieve greater standardisation of protocols based on these serum markers and to assess their predictive value in experimental infections for vaccine testing, as well as to determine the biological function of these proteins in the course of infectious diseases.

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