RESEARCH ARTICLE

Immune and acute phase response in pigs experimentally infected with H1N2 swine influenza virus

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Abstract

Acute phase proteins (APPs) and immune responses in pigs after experimental infection with H1N2 swine influenza virus (SwH1N2) were studied. Eight piglets were infected intranasally with SwH1N2. Four served as controls. Antibodies against swine influenza virus (SIV) were measured by hemagglutination inhibition assay. The proliferation assay was used to measure influenza-specific cell-mediated response. Hematological parameters were measured on a hematology analyzer. C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA) and pig major APP (Pig-MAP) concentrations in serum were measured using commercial ELISAs. Antibodies against SwH1N2 in the serum of infected pigs were detected from 7 dpi. SwH1N2-specific T-cell response was observed from 5 dpi. A significant drop in lymphocyte numbers and an increase in medium-sized cell (MID) counts with no accompanying leukopenia was observed in all infected pigs from 3 to 7 dpi. In infected pigs, concentrations of CRP, Hp and SAA increased significantly when the greatest amounts of virus were shed (from 1 to 3 dpi). The level of Pig-MAP remained unchanged during study. The significant positive correlation found between maximum concentrations of SAA in serum and lung scores, makes SAA a potentially useful indicator in experimental infection studies (e.g. vaccine efficiency investigations) or as a marker for disease severity, but to confirm this hypothesis more studies are needed.

Introduction

The acute phase response is a very complex reaction, involving local and systemic effects, and may be caused by a range of factors (Heegaard et al., 1998; Carroll et al., 2004; Murata et al., 2004; Díaz et al., 2005; Grau-Roma et al., 2009, Pomorska-Mól et al., 2011a, b). One of these effects corresponds to changes in the concentration of several plasma proteins, which are called acute phase proteins (APP). The clinical utility (e.g. discriminating between bacterial and viral infections, monitoring of treatment efficacy, prognostic markers) of APPs measurements has been extensively studied in human patients (Hogarth et al., 1997; Lau et al., 2006; Tsiakalos et al., 2009; Chai et al., 2010). A similar diagnostic value of APP has been proposed in veterinary medicine (Alava et al., 1997; Carroll et al., 2004; Murata et al., 2004). The APP response has been observed in common pig infections (Grau-Roma et al., 2009; Pomorska-Mól et al., 2011a, b) but it is not known whether infection with H1N2 swine influenza virus (SIV) also induces an APP response. Influenza virus infections are a familiar and important cause of respiratory disease in pigs (Olsen et al., 2006). Typical SI outbreaks are characterized by a rapid onset of high fever, loss of appetite, labored abdominal breathing and coughing. Mortality is low and recovery occurs within 7–10 days (Olsen et al., 2006). Human-avian reassortant H1N2 virus was first isolated from pigs in the UK in 1994 (Brown et al., 1995) and has a very complex history of origin. The virus appears to be a combination of the HA of a human H1N1 virus from the early 1980s and the NA of the swine H3N2 virus. The internal proteins are ‘avian-like’ as in the swine H1N1 and H3N2 viruses (Brown et al., 1998). In Poland, recent
serological investigations have confirmed an increased prevalence of H1N2 SIV subtype in Polish pigs. The seroprevalence increased from 0% in 2003 to 9.4% in 2009, and to 18.8% in 2011 (Markowska-Daniel & Kowalczyk, 2005; Van Reeth et al., 2008; I. Markowska-Daniel, A. Kowalczyk and K. Kwit, unpublished data).

The primary objective of this study was to assess whether experimentally induced infection, caused by emerging SIV subtype in Poland, H1N2, evokes C-reactive protein (CRP), haptoglobin (Hp), serum amyloid A (SAA) and/or pig major APP (Pig-MAP) responses in pigs. The second objective was to assess whether the correlation between concentrations of investigated APPs in serum and changes in the lungs, existed. This study also investigated the development of humoral and cellular immune responses during infection.

Materials and methods

Animals

Twelve piglets of France Hyrides FH900 line, c. 5 weeks old, were studied. Both sexes were sourced from high health status herd and, prior to the start of the study, were shown to be both influenza A virus and antibody [subtypes H1N1, H1N2, H3N2, pandemic (H1N1) 2009] negative by matrix (M) gene real time RT-PCR and hemagglutination inhibition assay (HI), respectively.

During the experiment, the piglets were housed in two isolated units, one for the control pigs and one for the infected pigs, in a containment level 3 facility. Animal use and handling protocols were approved by Local Ethical Commission (University of Life Sciences in Lublin, Poland).

Preparation of virus inoculum

SIV A/sw/Granstedt/IDT3475/2004 (subtype H1N2; hereafter referred to as SwH1N2), kindly provided by IDT (Germany), was used for the experimental infection. The virus concentration was evaluated in Madin–Darby canine kidney cells and stored at –80 °C until used. Virus titers were calculated using method of Reed & Muench (1938).

Experimental design

On day 0, eight piglets were inoculated with SwH1N2. Inoculations of 10^8.3 TCID50 of virus in 2 mL of phosphate-buffered saline (PBS) were given intranasally. Four mock-inoculated pigs (with PBS) served as control pigs.

To examine the events taking place at the early stages of infection with SwH1N2, three infected and one control piglet were euthanized at 3 days post infection (dpi). Remaining pigs were euthanized at 10 dpi. Pigs were sedated and then euthanized by injection of barbiturate in the ear vein. Necropsy was performed immediately after the animals were euthanized.

Clinical and pathological examination

Rectal temperatures were taken daily and clinical signs of disease were recorded. Pigs were observed and scored for respiratory signs as follows: for respiratory rate, 0 – normal; 1 – slightly elevated; 2 – moderately elevated, slight abdominal breathing and 3 – clearly elevated, distinct abdominal breathing; for nasal discharge, 0 – absent and 1 – present; for coughing: 0 – absent and 1 – present; for sneezing, 0 – absent and 1 – present. All scores were accumulated for a total clinical score of each individual pig (0–6). Rectal temperature was also measured daily. Fever was recorded when the rectal temperature was ≥ 40 °C.

Blood samples and nasal swabs were collected on 0 (challenge), 1, 2, 3, 5, 7 and 10 dpi. Serum was harvested after centrifugation and stored at −20 °C for further analyses. Complete necropsy was done on each animal, with special emphasis on the respiratory tract. Gross lung lesions were assessed for the presence or absence of pulmonary cranioventral multifocal consolidation and extent was recorded when present. Samples from lung (apical, middle and diaphragmatic lobes) and tracheas were collected aseptically and frozen at −80 °C until their use for viral RNA extraction.

Lung score

Lungs were assessed according to the scheme described by Christensen et al. (1999). If no changes were found in the lobe, it was scored as 0%. Changes in the right lung were scored as follows: apical lobe 10%, cardiac lobe 10%, diaphragmatic lobe 35% (in all, 55%). Changes observed in the left lung were scored as: apical lobe 5%, cardiac lobe 5%, diaphragmatic lobe 30%. Changes in the intermediate lobe were scored as 5%. All recorded scores were then added together to determine final visual lung score for each pig, ranging from 0% to 100%.

Laboratory examination

Swabs and tissue samples

The general swine influenza A real time RT-PCR method [the ‘perfect match’ matrix (M) gene real time RT PCR] was used for detection of SIV in nasal swabs, tracheas and lung samples, as described previously (Slomka et al., 2012 Federation of European Microbiological Societies Published by Blackwell Publishing Ltd. All rights reserved
Viral RNA was extracted from nasal swabs and tissues using the QIAamp Viral RNA extraction kit (Qiagen, Valencia, CA). RRT-PCR was performed using a one-step RT-PCR kit (Qiagen). The oligonucleotide sequences (probe and primers) were specific for the matrix gene region of known European swine influenza A viruses, as well as any swine infections that may be due to pandemic H1N1 2009 influenza A virus. Samples with a $C_t$ value < 30 were considered to be M gene-positive, samples with a $C_t$ value 30–35 were considered to be weak positive, and samples with $C_t$ value > 35 were considered to be negative.

**Hematological examinations**

Whole blood samples were analyzed for different leukocyte proportions and concentrations on an Abacus Junior Vet 5 hematology analyzer (Diatron, Hungary). Proportions of lymphocytes (LYM), MID and granulocytes (GRA) were calculated as a percentage of leukocyte concentration.

**Lymphocyte proliferation assay**

The T-cell proliferation assay to measure influenza-specific T-cell responses of pigs was performed at 0, 5, 7 and 10 dpi, as described for pseudorabies virus (Kimman et al., 1995). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from blood samples by centrifugation onto Histopaque 1.077 (Sigma) and were washed twice with PBS. The isolated PBMC were seeded in plastic vials at a density of 10^6 viable cells per vial in 1 mL medium (RPMI 1640 containing 10% fetal bovine serum, 2 mM l-glutamine and 1% antibiotic-antimycotic solution). For analysis of cellular responses, PBMC were restimulated in vitro with 50 μL of medium containing live SwH1N2 virus (titer 10^6.5 TCID₅₀ 50 μL⁻¹ and 256 hemagglutinin units). In control vials, the cells were incubated without the virus (mock-control) or with 5 μg mL⁻¹ of concanavalin A (Con-A; vitality control). All samples were analysed in triplicate.

After 72 h incubation at 37 °C in 5% CO₂ atmosphere, the cultures were pulsed with 0.5 μCi [³H]-thymidine (MP Biomedicals). After 18 h incubation, cells were harvested and the incorporated radioactivity was measured in a liquid scintillation counter (Tri-Carb 2500TR; Packard). Proliferation was expressed as the stimulation index (SI), calculated as the number of counts per minute (cpm) for SwH1N2-stimulated cells divided by the number of cpm for the mock-stimulated cells (in each case taking the mean of triplicate vials).

Based on the SI values of the control group (mean plus 3 × standard deviation), SI value ≥ 1.7 was considered to be positive.

**Serum analyses: hemagglutination inhibition assay**

Antibodies against SIVs were measured using an HI assay. The HI assay was performed according to the standard procedure, using 0.5% chicken erythrocytes and 4 HA units of strain SwH1N2 and, additionally, using other subtypes H1N1, H3N2 and pandemic (H1N1) 2009 (Swenson et al., 2008). All sera were tested in serial twofold dilutions, starting at 1 : 20. For estimates of the antibody prevalence, titers equal to or higher than 20 were considered positive.

**Serum analyses: APP determination in serum samples**

Commercial ELISAs were used to determine APP according to the manufacturers’ instructions (Pig CRP ELISA and Pig haptoglobin ELISA from Life Diagnostics, Inc.; PigMAP Kit ELISA from PigCHAMP Pro Europa S.A, Spain; Phase SAA Assay from Tridelta Development Ltd, County Kildare, Ireland). For all analyses, serum samples were tested in duplicate. Prior to analyses, serum samples were diluted as follows: 1 : 1000 for CRP, 1 : 35 000 for Hp, 1 : 500 for SAA and 1 : 1000 for Pig-MAP.

**Statistical analysis**

The obtained data were subjected to the Shapiro–Wilk’s test of normality and the Levene’s test of equal variances with STATISTICA 8.0 (StatSoft). To compare mean APP concentration, a nonparametric Kruskal–Wallis test with multiple comparisons for all pairs was used. For analysis of correlation, the Spearman rank correlation test was used. For all analyses, $P < 0.05$ was considered significant.

**Results**

**Clinical signs**

All infected pigs displayed mild clinical signs, including coughing, sneezing and nasal discharge. Six infected pigs showed transient abnormal rectal temperatures (> 40 °C) between day 1 and 3 post infection. In the control pigs, no clinical signs of any disease were seen and rectal temperatures were ≤ 40 °C. Rectal temperatures of infected pigs are presented in Fig. 1.

**Pathological examination**

Postmortem examination revealed typical lesions deriving from SIV infection in the lungs of all eight infected pigs. The mean lung score was 15 ± 4.79% (range 10–20%).
There were no significant differences in lesion scores between pigs necropsied at 3 dpi and those necropsied at 10 dpi.

**Hematological examination**

The results of the present study demonstrated that in SwH1N2-infected pigs, the overall number of leukocytes remained stable during the study and ranged from $16.06 \times 10^6$ mL$^{-1}$ to $19.12 \times 10^6$ mL$^{-1}$ (Kruskal–Wallis, $P > 0.05$; Fig. 2). In control pigs, the total number of leukocytes ranged from $16.65 \times 10^6$ mL$^{-1}$ to $18.67 \times 10^6$ mL$^{-1}$. The number of LYM decreased significantly (Kruskal–Wallis, $P < 0.05$) from 3 dpi and remained at the same level until the end of the study. The decrease was from $10.19 \pm 2.98 \times 10^6$ mL$^{-1}$ on day 0 to $6.1 \pm 2.66 \times 10^6$ mL$^{-1}$ on 3 dpi, and to $6.42 \pm 0.5 \times 10^6$ mL$^{-1}$ on 10 dpi. The percentages of LYM were the lowest between 3 and 7 dpi, ranging from 38% to 39%, whereas on day 0 the percentage of LYM reached over 50%. In control piglets, the mean concentration as well as percentage of LYM remained stable, ranging from $8.13 \times 10^6$ mL$^{-1}$ to $10.43 \times 10^6$ mL$^{-1}$ and from 48% to 54%, respectively. The absolute concentration of GRA remained stable in all piglets (infected and control) during the experiment, ranging from 7.81 to 9.95 $\times 10^6$ mL$^{-1}$. The percent of GRA (48–56%) did not change significantly after infection (Kruskal–Wallis, $P > 0.05$). A significant increase of MID cells (represented mainly by monocytes) was observed from 3 to 7 dpi (Kruskal–Wallis, $P < 0.05$). The lymphocyte : MID ratio was over 70 on 0 and 10 dpi, whereas from 3 to 7 dpi the ratio decreased to below 15 for both the absolute and the relative lymphocyte : MID ratio. The mean lymphocyte : MID ratio in control pigs ranged from 70 to 85 (Fig. 3).

**In vitro cellular response**

The mean SI values in control pigs and pigs from the experimental group before inoculation ranged from 0.68 to 1.03. Five days after infection, in three infected pigs an individual SI was higher than 1.7. From 7 dpi, the
remaining infected pigs (n = 5) developed an antigen-specific proliferation. The mean SI values (± SD) in infected piglets are presented in Fig. 4.

**Antibody response against SwH1N2**

All infected pigs exhibited SwH1N2-specific antibodies against hemagglutinin (anti-HA) in the serum between 7 and 10 dpi (Table 1). The uninfected pigs had no detectable HI serum antibodies.

**Presence of SIV in swabs and tissues**

An M gene RRT-PCR assay which was used to confirm the presence of SIV in the nasal swabs, revealed positive results from infected pigs between 1 and 7 dpi (Table 2). No SIV genetic material was found in the nasal swabs taken before inoculation and in the swabs taken from control pigs. No viral RNA was detected in swabs taken at 10 dpi. In the infected pigs euthanized at 3 dpi, the presence of SIV was confirmed in the trachea as well as the middle and apical lobes. In two piglets, the occurrence of SIV was also confirmed in the accessory lobes. No viral RNA was detected in the lungs at 10 dpi.

**Acute phase proteins**

The concentrations of CRP, Hp and SAA were significantly increased after infection, with mean maximum levels from days 1 to 2. The time course of mean APP concentrations (± SD) in infected and control pigs during the study period is presented in Fig. 5.

The mean concentration of CRP was significantly higher from 1 to 2 dpi in infected pigs as compared with control pigs (P < 0.05). The mean maximum concentration reached 46.76 ± 6.1 μg mL⁻¹ and was over 2.5-fold higher than before inoculation. From 3 dpi the CRP concentration decreased and did not differ significantly between control and infected pigs.

Pre-inoculation individual levels of Hp were found to be below 0.65 mg mL⁻¹, and the highest individual induced level reached above 3.2 mg mL⁻¹ (at 2 dpi). Changes in serum Hp concentrations were observed in all SwH1N2-infected pigs. The concentration of Hp had increased 24 h after inoculation, by which time the mean concentration of Hp was significantly higher than in the control pigs (P < 0.05). The mean peak level was over fourfold higher than the mean pre-inoculation concentration. The level of Hp remained significantly elevated up to 3 dpi. From 5 dpi the mean level of Hp did not differ significantly between the control and the infected pigs.

The mean concentration of SAA also increased significantly during the first 24 h after inoculation. A statistically significant increase of mean SAA concentration, as compared with the control pigs, was observed only on 1 and 2 dpi (P < 0.05). The mean peak level reached 29.49 ± 9.02 μg mL⁻¹ and was almost 10-fold higher compared with the level on day 0. From 3 dpi the SAA concentrations decreased and did not differ significantly between control and infected pigs. A positive correlation was found between the maximum concentration of SAA in serum and lung scores (Spearman R = 0.7233, P < 0.05; Fig. 6).

The mean concentrations of Pig-MAP in infected pigs ranged from 0.78 to 0.88 mg mL⁻¹ and did not differ significantly from those observed in control animals (P > 0.05).

**Discussion**

The present study characterized immune as well as CRP, Hp, SAA and Pig-MAP responses after intranasal infection with SwH1N2 virus during the first 10 dpi. In addition, the relationship between APP concentrations and pathological changes in the lungs were analyzed. Previous studies in pigs have described the kinetics of APP response in the course of various diseases (Heegaard et al., 1998; Sorensen et al., 2006; Grau-Roma et al.,
Measurements of APP have been used extensively for monitoring health status and disease progression, and in the evaluation of treatment efficacy (Carroll et al., 2004; Sorensen et al., 2006; Pomorska-Möl et al., 2011a). To our knowledge, APP response has not been studied previously in H1N2-inoculated pigs.

After infection of pigs with SwH1N2, various kinetics of responses could be identified within the APP tested. APPs CRP and SAA were significantly induced from 1 to 2 dpi, and Hp was significantly induced from 1 to 3 dpi. The concentration of Pig-MAP remained unchanged to the end of study. A significant rise in CRP, Hp and SAA concentrations was found before the specific antibodies and antigen-specific proliferation were detected. Antibodies against SwH1N2 in the serum of infected pigs were detected from 7 dpi, which is in accordance with previous reports for various subtypes of SIVs (Heinen et al., 2000; Van Reeth et al., 2006). Antigen-specific T-cell response was observed as early as 5 dpi, in three of five infected pigs. Starting from 7 dpi the significant antigen-specific response was seen in all infected animals. In general, T-cell proliferation was observed before antibodies against hemagglutinin were present.

The presence of over a fourfold increase in Hp and a 2.5-fold increase in CRP concentrations after inoculation with SwH1N2 places these markers in the category of moderate APP (Murata et al., 2004). SAA, with over 10-fold increase, could be placed in the category of major APP in pigs (Murata et al., 2004).

The cytokines and APPs associated with acute swine influenza in pigs were previously investigated by Barbé et al. (2011) after infection of pigs with the H1N1 subtype of SIV. The concentrations of CRP and Hp were analyzed during the first 5 dpi. In general, the serum concentrations of CRP and Hp peaked at 2 dpi. However, no
significant differences were found between infected and control pigs due to small number of pigs in the study. An increase of CRP and Hp was also reported by Brookes et al. (2010) during influenza in pigs caused by pandemic (H1N1) 2009 virus. The CRP levels peaked at 4 dpi with a mean maximum concentration about 32 µg mL⁻¹. The more protracted Hp responses peaked at 9–11 dpi, with the mean concentration on these days between 4 and 6 mg mL⁻¹ (Brookes et al., 2010). In our experiment the CRP levels peaked earlier and the mean maximum CRP concentration was higher (46.45 µg mL⁻¹). In the study by Brookes et al. (2010) Hp increased over threefold, in agreement with the data obtained in the present study. The very high concentration of Hp after infection had been induced observed by Brookes et al. (2010) could be a consequence of high basal concentration of this protein in pig serum (about 2 mg mL⁻¹). An increase of serum Hp had also been found earlier in ponies experimentally infected with the influenza virus (IV; Kent & Goodall, 1991).

The APP SAA has proven potentially useful as a bacterial infection marker. However, information about the SAA response in viral diseases, including influenza, is limited. An increase in serum SAA has been reported in humans and horses with influenza (Whicher et al., 1985; Hulten et al., 1999). Whicher et al. (1985) found that in humans after experimental influenza A virus infection the SAA concentration peaked at 3 dpi and was significantly different from that observed in controls. Similar to previous findings, Hulten et al. (1999) reported that equine SAA responds to equine influenza infection and that a positive correlation was found between the concentration of SAA in horses and clinical signs of the disease. The positive relationship between maximum concentration of SAA in serum of infected pigs and lung score was also observed in our study.

According to Parra et al. (2006), who investigated APP response in other viral disease (porcine reproductive and respiratory syndrome, pseudorabies), no significant response of Pig-MAP was seen. On the other hand, pigs
Response in pigs infected with H1N2 swine influenza virus

with PCV2 infection showed Pig-MAP concentrations significantly higher than those of controls (Parra et al., 2006). An explanation for this could be that PCV2 infection is characterized by systemic inflammation, not only local inflammation, with frequent co-infection with bacteria (Parra et al., 2006).

The hematological investigations showed a significant drop in lymphocyte numbers and an increase in MID counts, with no accompanying leukopenia, in all infected pigs. There was a 40% drop in mean number of LYM between 0 and 3 dpi, whereas the total number of white blood cells (WBC) remained unchanged. Additionally, an increase of over 500% in the mean number of MID cells was observed between 0 and 3 dpi. The mean number of LYM did not return to the mean pre-inoculated concentration; however, the mean number of MID cells returned to normal concentrations at 10 dpi. Previously, in adult human patients with influenza A, relative lymphopenia was found to be an early and reliable laboratory finding (Mohan et al., 2004; Cunha, 2008). Peripheral lymphopenia was also reported in humans infected with highly pathogenic avian influenza H5N1 (Imai et al., 2009). The induction of lymphocyte apoptosis by death receptor ligands may play a role in lymphopenia related to influenza infection (Zhou et al., 2006). In accordance with our findings, relative lymphopenia without leukopenia has previously been found in humans with positive test results for H1N1. In the field of human medicine, it has been suggested (Cunha et al., 2009) that relative lymphopenia might be a marker for H1N1 and thus could also be used to prioritize H1N1 PCR testing if the capability of the emergency department was exceeded (Cunha et al., 2009).

Our results indicated that concentrations of CRP, Hp and SAA increased significantly in pigs infected with H1N2, but these increases were smaller than reported previously for various bacterial pig diseases (Heegaard et al., 1998; Sorensen et al., 2006; Pomorska-Mol et al., 2011a, b). With the above information in mind, future studies should focus on the possibility of using APP as markers for the differentiation of bacterial and viral diseases in pigs under field conditions. The concentrations of CRP, Hp and SAA significantly increased before specific antibodies as well as antigen-specific proliferation were detected. More studies are also needed to evaluate whether relative and/or absolute lymphopenia might be a marker for H1N2 infection in pigs and whether relative and/or absolute lymphopenia also occurs with infection with other subtypes of influenza viruses.

The significant positive correlation found between maximum concentrations of SAA in serum and lung scores, makes SAA a potentially useful indicator in experimental infection studies (e.g. vaccine efficacy investigations) or as a marker for disease severity. More studies are needed to confirm this hypothesis.

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References


