DEVELOPMENT OF EARLY HUMORAL AND CELL-MEDIATED IMMUNITY IN PIGLETS WITH EXPERIMENTALLY INDUCED SUBCLINICAL SWINE INFLUENZA

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Abstract

Development of early immune response in piglets with subclinical swine influenza was investigated. Fourteen, seronegative piglets were used. Ten of them were infected intranasally with swine influenza virus (SIV) H1N1 subtype. Temperature and clinical signs were assessed daily. Leukocyte proportions and concentrations were analysed on a haematology analyser. Antibodies against SIVs were measured by haemagglutination inhibition assay. To measure influenza-specific cell-mediated immunity (CMI), the proliferation assay was performed. The real time reverse transcription PCR method was used for detection of SIV. No relevant respiratory or systemic clinical signs were observed. The presence of SIV RNA in nasal swabs from all infected piglets was confirmed between 2 and 5 dpi. The overall number of leukocytes did not differ during the study. The number of medium-sized cells (MID) was significantly higher on 2 and 4 dpi, as compared to day 0 level. The percentage of lymphocytes decreased from 74% on day 0 to 67.06% on 4 dpi, while the percentage of MID significantly increased at the same time. In control pigs no significant changes were observed. All infected pigs exhibited specific antibodies between 7 and 10 dpi. Specific CMI was observed before specific antibodies were present. Results of our research indicate that kinetics of the humoral and CMI response during subclinical infection is similar to that observed in clinical form of the disease.

Key words: piglets, H1N1 swine influenza virus, subclinical infection, immune response.

Swine influenza (SI) is a highly contagious respiratory disease caused by various subtypes of swine influenza viruses (SIVs) (10, 15, 19). At present, three main subtypes of SIVs are circulating in the swine population throughout the world: H1N1, H1N2, and H3N2, (1, 14, 15, 21). Typical SI outbreaks are characterised by a rapid onset of high fever, loss of appetite, laboured abdominal breathing, and coughing. Mortality is low and recovery occurs within 7-10 d (10, 19). However, the infection is much more frequent than the disease. Infection with swine influenza H1N1 virus is frequently subclinical and typical signs are often demonstrated only in 25% to 30% of a herd (1, 2).

While data regarding immune response during acute course of SI in pigs are available, there is lack of data concerning development of humoral and T-cell response in pigs with subclinical infection. Therefore, the aim of the present study was to investigate the development of humoral and cell-mediated immunity in piglets with experimentally induced subclinical swine influenza (subSI), caused by H1N1 subtype of SIV. Additionally, the changes in the white blood cell populations during infection were assessed.

Material and Methods

Animals. Fourteen 7-week-old piglets of 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, pH1N12009) negative by Matrix (M) gene real time reverse transcription PCR method (RRT-PCR) and haemagglutination inhibition assay (HI), respectively. Animal use and handling protocols were approved by the Local Ethical Commission. Infected piglets were housed separately from control ones.

Preparation of virus inoculum and challenge. Swine influenza virus A/sw/Poland/KPR9/2004 (subtype H1N1) (hereafter referred to as SwH1N1), which had been isolated from the lungs of fattening pig, was used for experimental infection. The stock used for inoculation represented the third passage in eggs. The virus titre was evaluated in Madin-Darby canine kidney
(MDCK) cells. Ten piglets were inoculated intranasally with 3 ml of viral suspension containing $10^{3.3}$ TCID$_{50}$ SwH1N1/mL. Four piglets inoculated with PBS served as controls.

**Experimental design.** On day 0, ten piglets were inoculated with SwH1N1. In order to examine the events taking place at the early stages of infection with SwH1N1, two infected and one control piglets were euthanized on days 2 and 4 post infection (dpi). The remaining piglets were euthanized on 10 dpi. Necropsy was performed immediately after the animals were euthanised.

**Clinical and postmortem examination.** Rectal temperatures were assessed daily and clinical signs of disease were recorded. Fever was defined as body temperature of $>$40°C.

Blood samples 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. Nasal swabs were taken on 2, 3, 4, 5, 7, and 10 dpi. 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. Lung samples and tracheas were collected aseptically and frozen at -80°C until their use for viral RNA extraction.

**PCR.** The general swine influenza A RRT-PCR method (the “perfect match” M gene RRT PCR) was used for detection of SIV in nasal swabs, tracheas and lung samples, as described previously (20). Samples with Ct value <30 were considered to be M gene positive, samples with Ct value 30-35 with sigmoidal/logarithmic appearance were considered to be weakly positive, samples with Ct value >35 were considered to be negative.

**Haematological examination.** Whole blood samples were analysed for different leukocyte proportions and concentrations on a Abacus Junior Vet 5 haematology analyser (Diatron, Hungary). Proportions of lymphocytes, medium-sized cells (MID) (represented mainly by monocytes), and granulocytes were calculated as a percentage of leukocyte concentration.

**Humoral immune response.** Antibodies against SIVs were measured using a haemagglutination inhibition (HI) assay. The HI assay was performed according to the standard procedure, using 0.5% chicken erythrocytes and 4HA units of strains H1N1 used for inoculation (SwH1N1 virus). All sera were tested in serial twofold dilutions, starting from 1:20. To estimate the antibodies prevalence, titres equal or higher than 20 were considered positive.

**Proliferation assay (PA).** The proliferation assay, used to measure influenza-specific T-cell responses was performed on 0, 2, 4, 7, and 10 dpi, as described for pseudorabies virus (9). Briefly, PBMC were isolated from blood samples by centrifugation on Histopaque 1.077 (Sigma, USA) and were washed twice with PBS. The isolated PBMC were seeded in plastic vials at a density of $1 \times 10^8$ viable cells per vial in 1 ml medium (RPMI 1640 containing 10% foetal bovine serum, 2 mM L-glutamine and 1% of antibiotic-antimycotic solution). PBMC were restimulated in vitro with 50 µl of medium containing live SwH1N1 virus (titer $10^6$ TCID$_{50}$). In control vials, the cells were incubated without the virus (mock-control) or with 5 µg/mL of concanavalin A (Con-A) (vitality control). All samples were analysed in triplicate.

After 72 h of incubation at 37°C in 5% CO$_2$ atmosphere, the cultures were pulsed with 0.5 µCi [³H]-thymidine (MP Biomedicals, USA). After 18 h of incubation, the cells were harvested and the incorporated radioactivity was measured in a liquid scintillation counter (Tri-Carb 2500TR, Packard, USA). Proliferation was expressed as a stimulation index (SIX). The SI was calculated as the number of counts per minute (cpm) of SwH1N1 restimulated PBMC divided by the number of cpm of the mock-control-stimulated cells (in each cases taking mean of triplicate vials).

Based on the SIX values observed before infection (day 0) and in control animals, SIX values pointing to influenza-specific proliferation were established (as mean plus 3 x standard deviation). SIX values $\geq$1.55 were considered to be positive.

**Statistical analysis.** The obtained data were subjected to the W. Shapiro-Wilk’s test of normality and the Levene’s test of equal variances. A nonparametric Kruskal-Wallis test with post hoc multiple comparisons for comparison of all pairs was used for comparison of investigated parameters. Differences with $\alpha <0.05$ were considered as significant. All calculations were performed with the Statistica 8.0 (Statsoft, Poland) computer programme.

**Results**

**Clinical signs.** No relevant respiratory or systemic clinical signs were observed in infected piglets. However, five animals showed transient abnormal rectal temperatures ($>$40°C) between days 1 and 3 post infection. The general condition of the piglets and feed intake were unchanged during the whole period of the experiment.

**Postmortem examination.** Postmortem examination revealed typical lesions, deriving from SIV infection, in the lungs of eight out of ten infected piglets. One pig euthanised on 2 dpi and one euthanised on 4 dpi had no macroscopic changes in the lungs.

**Presence of RNA of SIV in swabs and tissues**

No SIV genetic material was found in the nasal swabs taken before inoculation. M gene RRT-PCR assay, used to confirm the presence of SIV in the nasal swabs revealed positive results from all infected pigs between 2 and 5 dpi. No viral RNA was detected in swabs on 7 and 10 dpi. The presence of SIV RNA was also analysed in the trachea and lungs. In all infected pigs, euthanised on 2 and 4 dpi, the presence of SIV was confirmed for both the trachea and lungs. No viral RNA was detected in the lungs on 10 dpi as well as in the lungs of uninfected pigs.

**Haematological examination.** The results of the presented study demonstrated that the overall
number of leukocytes did not differ significantly during the study and ranged from 19.23 to 21.28 \times 10^9/L (Kruskal-Wallis, P>0.05). The absolute and relative number of lymphocytes, MID, and granulocytes are shown in Figs 1 and 2, respectively. The number of MID was significantly higher (Kruskal-Wallis, P<0.05) on 2 and 4 dpi, as compared to day 0 level. The increase was from 0.1 ±0.01 on day 0 to 0.46 ±0.16 and to 1.06 ± 0.1, on 2 and 4 dpi, respectively. The percentage of lymphocytes decreased from 74 ±1.84% on day 0 to 67.06 ±2.39% on 4 dpi (Kruskal-Wallis, P<0.05), while the percentage of MID significantly increased at the same time. The absolute and relative size of granulocytes remained stable during the experiment (Kruskal-Wallis, P>0.05). The absolute lymphocyte/MID ratio (Fig. 3.) decreased from 2 dpi and remained significantly lower to 4 dpi (Kruskal-Wallis, P<0.05). It was also true for relative lymphocyte/MID ratio. In control piglets no significant changes were observed.

**Antibody response against SwH1N1.** All infected piglets exhibited antibodies against haemagglutinin (anti-HA) in the serum, between 7 and 10 dpi. (Table 1). Sera from piglets in the control group had no antibody titres (<20 HI titre). All uninfected piglets remained seronegative to SwH1N1 till the end of the experiment.

**In vitro cellular response.** The mean SIx values in control piglets and piglets from experimental group before inoculation (0 dpi) ranged from 0.71 to 1.16. Four days post infection in five out of eight infected piglets an individual SIx was higher than 1.55. Starting from 7 dpi all infected piglets developed an antigen-specific proliferation. The mean SIx values (+SD) in infected piglets are shown in Fig. 4.

**Discussion**

In the presented study, the development of antigen-specific humoral and cell–mediated immunity, and changes in the white blood cell populations in piglets with experimentally induced subSI were investigated during the first 10 dpi.

![Fig. 1. The absolute number of lymphocytes, MID, and granulocytes in infected piglets.](image1)

* P<0.05, ** P<0.01, *** P<0.001 - significance of differences as compared to day 0 level.

![Fig. 2. The relative number of lymphocytes, MID, and granulocytes in infected piglets.](image2)

* P<0.05, ** P<0.01, *** P<0.001 - significance of differences as compared to day 0 level.
In the present study, a significant decrease in relative number of lymphocytes and relative and absolute number of MID, with no accompanying leukopenia, were found between 2 and 4 dpi in all infected piglets. There was almost a 10% drop in the mean relative number of lymphocytes between 0 and 4 dpi, while total number of white blood cells remained unchanged. Additionally, an over 300% increase in the mean number of MID cells was observed between 0 and 2 dpi. On 4 dpi, the mean number of MID was up to 10 times higher than before infection. The mean number of MID and percentage of lymphocytes returned to normal values after 7 dpi. Previously, relative lymphopenia has been found to be an early and reliable laboratory finding of adult human patients with influenza A (3, 18).

In humans with positive test results for H1N1 a relative lymphopenia is common, but leukopenia is not present, which is in accordance to our findings. Induction of lymphocyte apoptosis by death receptor ligands including TNF-α and TNF-related apoptosis-inducing ligand (TRAIL) may play an important role in lymphopenia associated with influenza infection (22). It was proposed in human medicine that relative lymphopenia might be a marker for H1N1, and thus it could also be used to prioritise H1N1 PCR testing, if the emergency department’s ability is exceeded (4). The peripheral lymphopenia was also reported in humans infected with highly pathogenic avian influenza H5N1 (7). More studies are needed to evaluate if the same diagnostic value of drop in lymphocyte relative number may be proposed in pigs.

Increasing number of monocytes was previously reported in human patients with H1N1 virus infection (17). Monocytes and macrophages appear to play a protective role during influenza virus infection, as it was shown on a mice model (13). Blood monocytes, or macrophages derived from blood monocytes, recruited to the lungs, participate in the early host response to influenza. An important beneficial activity of monocytes/macrophages during influenza infection is the phagocytosis of virus-infected apoptotic cells, which correlates with clearance of the virus from the lungs (5). Depletion of macrophages from pigs has been shown to impair the response to influenza virus (8).

The adaptive immune response helps to restrict the viral spread, eradicate virus, and finally to establish a memory response resulting in a long-lived resistance to re-infection with homologous virus; however, it requires some days to be effective. Influenza infection induce both systemic and local antibodies, as well as cytotoxic T cell responses (11, 19). Serum HI antibodies are the most commonly measured to determine the level of protection against influenza. Usually, they can be detected from 7-10 dpi. Peak titres are usually seen between 14 and 21 dpi (6, 11).

In the present study, the HA-specific antibodies in the serum of piglets with subclinical infection were also detected from 7 dpi, and the HI titres increased over time.

Regarding the cellular immune response, our result showed that SIV – specific proliferation appeared
very early, on 4 dpi (in five out of eight infected piglets). Starting from 7 dpi an antigen-specific response was noted in all infected animals and increased over time. This evolution generally agrees with the result of Larsen et al. (11). These authors found that antigen-specific T-cell response in pigs with acute influenza could be detected from 7 dpi reaching peak levels on 14 dpi. T lymphocyte influenza-specific responses in humans also peak at about day 14 post infection (12). Cell mediated immune response plays a role in recovery from influenza infection and may prevent influenza-related complications (19). Moreover, the level of SIV-specific T lymphocytes correlates with the speed of viral clearance from the respiratory tract (16).

In summary, this study defines the development and kinetics of early immune responses to H1N1 influenza virus during subclinical infection of pigs. Results of our research indicate that kinetics of the humoral as well as T-cell immune response during subclinical infection is similar to that observed in the clinical form of the disease (11). The T-cell specific immunity was observed before specific antibodies were present. More studies are needed to evaluate the significance of our findings on a decrease in the percentage of lymphocytes with corresponding increase in absolute and relative number of monocytes in the diagnostics of influenza in pigs.

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**References**