PHAGOCYTIC AND OXIDATIVE BURST ACTIVITY OF BLOOD PHAGOCYTES IN STREPTOCOCCOSIS AND MIXED RESPIRATORY INFECTIONS IN SWINE

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Abstract

The study aimed at assessing the parameters of the innate cellular response in streptococcosis and mixed respiratory infections in swine. Flow cytometry was used to determine the phagocytic and oxidative burst activity of neutrophils and monocytes. In Streptococcus suis infection, monocytosis was accompanied by a lower percentage of all phagocytizing cells in comparison to the control group, and a lower respiratory burst activity of monocytes was observed. In Streptococcus suis, Mycoplasma hyopneumoniae, and porcine reproductive and respiratory syndrome virus infection, the percentage of phagocytizing neutrophils and the percentage of neutrophils that succumbed to an oxidative burst were lower than in the control group, but the mean fluorescent intensity was higher in both tests. The oxygen burst activity of monocytes was also higher.

Key words: swine, Streptococcus suis, Mycoplasma hyopneumoniae, PRRSV, phagocytosis, oxidative burst.

Mixed infections are a widespread cause of respiratory diseases of swine. The common pathogens associated with porcine respiratory disease complex are porcine reproductive and respiratory syndrome virus (PRRSV), Mycoplasma hyopneumoniae (M. hyopneumoniae), and Streptococcus suis (S. suis) (8, 13, 17). The important risk factors of development of the disease complex are poor animal husbandry and farming system (12). The preliminary study showed that the prevalence rate of the infection caused by more than one infectious agent was 91% (18). Identification of the cellular mechanisms in the immune response to the infectious agents seems essential. It may advance our understanding of the course of the infection and help in evaluation of prognosis and treatment of the disease. To date, the majority of studies on the respiratory infections in swine was experimental and focused on a single pathogen only. Data obtained from studies of other disease complexes showed differences in the immune reaction to single infections and mixed infections, which occur under field conditions (11, 19). Flow cytometry and other modern techniques provide technical tools to assess complex immune response in mixed infections. New methods are constantly being developed, e.g. the interaction between S. suis and swine phagocytes were investigated using the green fluorescent protein as a specific marker (6). During our previous study we evaluated the acquired immune response to the infectious agents of respiratory diseases of swine (9).

The aim of the present experiment was to assess the effect of streptococcosis and mixed respiratory infections in swine on phagocytosis, as well as on the oxidative burst activity of neutrophils and monocytes.

Material and Methods

Animals. The study was conducted in three pig herds. The farms were selected on the basis of previously obtained bacteriological and serological data. The animals were isolated and not treated with antibiotics. A total number of 21 piglets, 12-15 weeks of age, were enrolled. The study included three groups: group 1 (control group) - seven clinically healthy pigs, free of S. suis, M. hyopneumoniae, and PRRSV infection; group 2 - seven pigs with clinical signs of streptococcosis and confirmed S. suis serotype 2 infection, but free of M. hyopneumoniae and PRRSV infections; group 3 - seven pigs with nonspecific clinical signs and confirmed mixed S. suis, M. hyopneumoniae, and PRRSV infection.
Bacteriological and serological examination. The lung tissue was sampled and bacteriological culture for the presence of *S. suis* was performed. PCR was used to confirm the diagnosis (7). ELISA was also carried out to detect antibodies against PRRSV (Idexx) and *M. hyopneumoniae* (Dako). The examinations were done in the National Veterinary Research Institute in Pulawy.

Clinical examination. The assessment of clinical signs was performed every day.

Sample collection. At the peak of clinical signs, peripheral blood was collected from the *vena cava cranialis* into the tubes containing EDTA-3K and lithium heparin (Medlab, Poland).

Flow cytometry analysis. The leucogram was determined by haematology analyser (Diatron Abacus, Austria) using EDTA-preserved whole blood samples. Cytometric analysis was performed in heparinised whole blood by FACSCalibur flow cytometer (Becton Dickinson, USA) and CellQuest software. Neutrophil and monocyte phagocytosis and respiratory burst activity were determined by Phagotest and Bursttest (Orpegen Pharma, Germany).

Phagotest. Aliquots of 100 µl of blood were incubated on ice for 10 min. Then FITC (fluorescein isothiocyanate) - labelled opsonised *E. coli* was added. The test was carried out with own modification – the procedure was adjusted to the pig body temperature – the samples were incubated for 10 min at 39.5°C in a water bath (vs. 37.5°C according to manufacturer’s instruction), the negative control sample remained on ice. The phagocytosis was stopped by placing the samples on ice and quenching solution was added. The erythrocytes were lysed. After washing, supernatant was discarded and leukocyte DNA was stained. The percentage of neutrophils and monocytes, which had ingested FITC-labelled *E. coli*, and their fluorescence intensity (number of bacteria per cell) were measured by flow cytometer.

Bursttest. Samples of blood (100 µl each) were placed on ice. Washing solution – negative control, fMLP (N-formyl-Met-Leu-Phe) – low control, PMA (phorbol 12-myristate 13-acetate) – high control and opsonised *E. coli* suspension were added to the tubes. The samples were incubated at 39.5°C. Then dihydrorhodamine (fluorescence probe for detection of intracellular reactive oxygen species – ROS) was added. The whole blood samples were lysed, the samples were centrifuged, and then the leukocyte DNA was stained after removing the supernatant. Flow cytometer was used to determine the number of cells expressing the fluorescence of rhodamine (percentage of ROS producing cells) and mean fluorescence intensity (amount of ROS produced per cell). Monocytes were stimulated by *E. coli* only (15).

Statistical analysis. Statistical analysis of the data was performed using STATISTICA 6.0. The mean and standard deviation were calculated. Mann-Whitney U test was applied to compare the significant differences between groups.

Results

Clinical signs. No clinical signs of the disease were observed in pigs in group 1 (control group). Pigs in group 2 (swine infected with *S. suis*) demonstrated high fever, ataxia, lameness, synovial effusion, cough, and mild anappetence. In group 3 (mixed *S. suis, M. hyopneumoniae*, and PRRSV infection) clinical signs such as: prolonged fever, cough, nasal discharge and ataxia were present. Additionally, appetite and body weight varied greatly between individuals. The decrease in weight gain was higher in group 3 than in group 2.

Phagocytic activity. The percentage of phagocytising cells (neutrophils and monocytes) was markedly lower in group 2 compared to the control pigs. In group 3 the percentage of phagocytising neutrophils and monocytes was significantly lower in comparison to both control group and *S. suis* infected swine; however, the mean fluorescence intensity was markedly higher. Detailed data are presented in Table 1.

Respiratory burst activity. It was found that in group 2, the percentage of neutrophils with oxidative burst activity after fMLP stimulation was significantly higher and the cellular reactive oxygen species level was higher than in control animals. Statistically significant differences were observed in the intensity of granulocyte oxidative metabolism after stimulation with *E. coli* – mean fluorescence intensity was significantly lower in comparison with control. In group 3, the percentage of neutrophils with respiratory burst activity was significantly lower in comparison with groups 1 and 2, but the mean fluorescence intensity was significantly higher, as it is shown in Table 2.

The percentage and the mean fluorescence intensity of monocytes with oxidative burst after *E. coli* stimulation was significantly lower in group 2. In group 3, there was no statistically significant difference in the percentage of monocytes producing ROS, but the level of reactive oxygen species was significantly higher in comparison with controls and group 2. Detailed data are presented in Table 3.

Discussion

This study demonstrated that different mechanisms of innate cellular immunity are involved in streptococcal infection and mixed respiratory infection. It has been suggested that PRRSV has an immunosuppressive effect. Feng et al. (4) suggested that PRRSV makes piglets more susceptible to infection. On the contrary, Van Alstine et al. (22) reported that PRRSV did not exacerbate *M. hyopneumoniae* infection, or, according to Albina et al. (1), may even have stimulating effects on the pig immune system during the phase of long-lasting infection. Effects of pathogens on porcine phagocytes were examined in several surveys (14, 16). The results of one study indicated that PRRSV infection could cause adverse alterations in morphology and function of alveolar macrophages and may potentially predispose pigs to secondary pulmonary infections (2).
Table 1
Result of the analysis of the phagocytic activity of neutrophils and monocytes of swine peripheral blood from the control (1), \textit{S. suis} infected (2), and \textit{S. suis}, \textit{M. hyopneumoniae}, and PRRSV infected (3) groups (mean ± SD)

<table>
<thead>
<tr>
<th>Group</th>
<th>Neutrophils (10^9/L)</th>
<th>Phagocyting cells (%)</th>
<th>Fluorescence intensity (FU)</th>
<th>Monocytes (10^9/L)</th>
<th>Phagocyting cells (%)</th>
<th>Fluorescence intensity (FU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – control (n = 7)</td>
<td>7.75 ± 1.73</td>
<td>44.0 ± 1.96</td>
<td>229.50 ± 27.47</td>
<td>0.88 ± 0.16</td>
<td>55.75 ± 2.25</td>
<td>152.50 ± 15.99</td>
</tr>
<tr>
<td>2 – \textit{S. suis} (n = 7)</td>
<td>4.40 ± 0.74</td>
<td>26.5 ± 5.17*</td>
<td>193.80 ± 12.92</td>
<td>1.41 ± 0.10*</td>
<td>12.25 ± 1.55*</td>
<td>169.25 ± 21.38</td>
</tr>
<tr>
<td>3 – \textit{S. suis}, \textit{M. hyopneumoniae}, PRRSV (n = 7)</td>
<td>8.57 ± 1.70</td>
<td>13.30 ± 2.66*</td>
<td>2762 ± 233.9*</td>
<td>1.49 ± 0.44</td>
<td>2.23 ± 0.53*</td>
<td>867.50 ± 93.84*</td>
</tr>
</tbody>
</table>

* P<0.05 in comparison to control group; \(^A\) P<0.05, \(^B\) P<0.01 in comparison to group 2.

Table 2
Result of the analysis of the oxygen metabolism of neutrophils of swine peripheral blood from the control (1), \textit{S. suis} infected (2), and \textit{S. suis}, \textit{M. hyopneumoniae} and PRRSV infected (3) groups (mean ± SD). Activators: fMLP (N-formyl-Met-Leu-Phe), PMA (phorbol 12-myristate 13-acetate), and \textit{E. coli}

<table>
<thead>
<tr>
<th>Group</th>
<th>fMLP</th>
<th>PMA</th>
<th>\textit{E. coli}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidised cells (%)</td>
<td>Fluorescence intensity (FU)</td>
<td>Oxidised cells (%)</td>
<td>Fluorescence intensity (FU)</td>
</tr>
<tr>
<td>1 – control (n = 7)</td>
<td>37.50 ± 2.10</td>
<td>45.25 ± 1.80</td>
<td>57.75 ± 9.96</td>
</tr>
<tr>
<td>2 – \textit{S. suis} (n = 7)</td>
<td>71.50 ± 3.70*</td>
<td>62.50 ± 4.77*</td>
<td>82.0 ± 3.67</td>
</tr>
<tr>
<td>3 – \textit{S. suis}, \textit{M. hyopneumoniae}, PRRSV (n = 7)</td>
<td>1.37 ± 0.78*</td>
<td>1.042.0 ± 202.92*</td>
<td>19.84 ± 4.81*</td>
</tr>
</tbody>
</table>

* P<0.05, in comparison to control group; \(^A\) P<0.05, in comparison to group 2.

Table 3
Result of the analysis of the oxygen metabolism of monocytes of swine peripheral blood from the control (1), \textit{S. suis} infected (2), and \textit{S. suis}, \textit{M. hyopneumoniae} and PRRSV infected (3) groups (mean ± SD). Activator: \textit{E. coli}

<table>
<thead>
<tr>
<th>Group</th>
<th>Oxidised cells (%)</th>
<th>Fluorescence intensity (FU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – control (n = 7)</td>
<td>3.50 ± 0.65</td>
<td>26.25 ± 3.86</td>
</tr>
<tr>
<td>2 – \textit{S. suis} (n = 7)</td>
<td>1.25 ± 0.25*</td>
<td>18.25 ± 0.48*</td>
</tr>
<tr>
<td>3 – \textit{S. suis}, \textit{M. hyopneumoniae}, PRRSV (n = 7)</td>
<td>2.98 ± 0.85</td>
<td>419 ± 99.07*</td>
</tr>
</tbody>
</table>

* P<0.05 in comparison to control group; \(^A\) P<0.05 in comparison to group 2.

Other research detected that the phagocytosis-suppressive effects of PRRSV act by reducing the total number of alveolar lung macrophages (10). PRRSV infection reduces the bactericidal ability of pulmonary intravascular macrophages and may have a detrimental effect on both, pulmonary intravascular and alveolar macrophages (20).

The presented study revealed that in pigs infected with \textit{S. suis} as well as in pigs with mixed respiratory infections, the percentage of phagocytizing cells was lower in comparison with the control group. Only in swine with mixed respiratory infection, the number of FITC-labelled opsonised \textit{E. coli} phagocytised by neutrophils and monocytes was higher, and in consequence, the mean fluorescent intensity was higher as well. This result seems to meet the general principle that the increase in the number of phagocytised bacteria compensates for the reduced percentage of phagocytizing cells (5, 21). On the contrary, the results of pigs infected with \textit{S. suis} only contradict the general principle.

The respiratory burst, which generates reactive oxygen species, is one of the main bactericidal mechanisms of phagocytizing cells. Analysis of the oxidative burst of peripheral blood neutrophils showed that only in swine with mixed infection, the percentage of neutrophils undergoing a respiratory burst was lower in comparison with the control group, and the mean fluorescent intensity was higher. The changes correspond to the alteration of their phagocytic activity. In \textit{S. suis} infected swine the analysis showed lower percentage of phagocytic monocytes as well as lower mean fluorescent intensity. The alterations were
accompanied by monocytosis. The result confirmed monocytes involvement in the immune response against the infection. There is no bone marrow reserve pool of monocytes comparable with the large reserve pool of granulocytes. Mature monocytes leave the bone marrow soon after their formation (3, 23). Taking these facts into consideration we also hypothesised that S. suis infection cause a release of immature monocytes from the bone marrow into the blood, or suppression of monocytes function. In group 3 (swine with mixed respiratory infection) the formation of intracellular oxidative products by monocytes was higher than in the control group (the mean fluorescent activity was higher).

Oxidative burst, which occurred in neutrophils was higher after FMLP stimulation, which may suggest a huge functional reserve of neutrophils. In our study, E. coli was the strongest activator of the respiratory burst in neutrophils of healthy pigs and PMA was the strongest in infected swine.

Summarising, we proved the involvement of different mechanisms of innate cellular response in monoinfection compared to the polyaethiological disease.

References