Influence of bivalent vaccine Miloxan (Clostridium perfringens type C,D and Clostridium oedematiens type B) and the C3 genotypes on lysozyme and complement concentrations in dairy sheep

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SUMMARY

The aim of this experiment was to study the influence of vaccination against Clostridium perfringens type C,D and Clostridium oedematiens type B and the C3 genotypes in dairy sheep on lysozyme and alternative pathway of complement activation (APCA) concentrations. Thirty-four dairy sheep [Stara Zagora x East-Friesian and (Stara Zagora x East-Friesian) x Blackhead Pleven breeds], were used belonging to the four principal genotypes: FS genotype (10 sheep), FF genotype (5 sheep), SS genotype (10 sheep) and F7S genotype (9 sheep). The animals were vaccinated with Bulgarian bivalent vaccine Miloxan (Clostridium perfringens type C,D and Clostridium oedematiens type B) after a baseline blood sampling for the determination of their immune status. The second analysis was performed 21 days after. Prior to the vaccination, the lysozyme concentrations and APCA activity between the C3 genotypes were not significantly different. After the vaccination, the lysozyme concentrations were increased, particularly for the SS genotype compared to the FS genotype (p<0.05). In the same way, vaccination induced increases of APCA values except for the SS genotype sheep. Maximal APCA values were observed in the SS genotype before vaccination, whereas following the vaccination, the FF genotype sheep presented maximal APCA activities, and the post vaccination APCA in the FF genotype was higher than the prevaccination APCA in the SS genotype (p=0.05). The vaccination against above bacteria and the C3 genotype affiliation influenced significantly lysozyme concentrations and the alternative pathway of complement activation in sheep.

Keywords : lysozyme - complement - sheep - vaccination.

Introduction

The effect of vaccinations on natural immunity is not fully elucidated. Several authors report adverse consequences following single or dual immunizations. ELKONIN [5] stated that following vaccinations of rabbits against Salmonella Breslau and Salmonella Tiphymurium, the titres of blood lysozyme exhibited a short-time increase but thereafter, decreased significantly for a long time. KRAVCHEKNO [8] gave evidence that the diphteria and tetanus pertussis toxoids (DTP) vaccine, and the bacille Calmette-Guérin (BCG) vac-
cine increased the systemic sensibility for 6 to 8 weeks. The same author has also reported that the application of an anti-
rabies vaccine to guinea-pigs, prevaccinated with pertussis vaccine, resulted in increased morbidity and death rate in animals for 6 weeks, whereas the control groups receiving either antirabies or pertussis vaccines remained in good health. GORSHUNOV A [7] having studied the vaccinal pre-
parations routinely used in humans (DTP and BCG) and reported that they consistently provoked changes in the non-
specific systemic resistance to a number of antigens, viral infections and respiratory diseases.

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Materials and methods

ANIMALS

Thirty-four dairy sheep [Stara Zagora x East-Friesian and (Stara Zagora x East-Friesian) x Blackhead Pleven breeds], were used in this study and were belonging to the four principal genotypes: FS genotype (10 sheep), FF genotype (5 sheep), SS genotype (10 sheep) and F7S genotype (9 sheep). The animals were vaccinated with Bulgarian bivalent vaccine Miloxan (Clostridium perfringens type C.D and Clostridium oedematiens type B) and the C3 genotypes in dairy sheep on lysozyme and APCA concentrations.

METHODS

Serum lysozyme concentrations were determined according to the method of LIE [11]. Twenty mL of 2% agarose (ICN, UK) dissolved in phosphate buffer (0.07 M Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>, pH = 6.2) were mixed with 20 mL suspension of 24 hours culture of Micrococcus lysodeicticus at 67°C. This mixture was poured out in Petri’s dish (14 cm diameter). After solidifying at room temperature 32 wells were made (5 mm diameter). Fifty microliters of undiluted sera were poured out in each well. Eight standard dilutions (from 0,025 to 3,125 µg/mL) of lysozyme (Veterinary Research Institute, Veliko Tarnovo) were used in the same quantity as well. The samples were incubated for 20 hours at 37°C and lytic diameters were measured.

The alternative pathway of complement activation (APCA) was studied by the method of SOTIROV [13]. Each serum sample was first diluted by mixing 100 µL serum with 350 µL veronal - veronal Na buffer (in final concentrations: 146 mM NaCl, 1.8 mM 5,5-diethylbarbituric acid sodium salt, 3.2 mM 5,5-diethylbarbituric acid, 1 mM EGTA and 0.8 mM MgCl<sub>2</sub>). In U bottomed plates (Flow Laboratories, UK), 7 other dilutions from each diluted serum were again prepared in veronal-veronal Na buffer: 80 µL diluted serum + 20 µL buffer, 70 µL diluted serum + 30 µL buffer, 60 µL diluted serum + 40 µL buffer, 50 µL diluted serum + 50 µL buffer, 40 µL diluted serum + 60 µL buffer, 30 µL diluted serum + 70 µL buffer and 20 µL diluted serum + 80 µL buffer. The final serum dilutions were respectively 8/45, 7/45, 6/45, 5/45, 4/45, 3/45 and 2/45. Then, 50 µL buffer and 100 µL of 1% rabbit erythrocyte suspension were added to each well. After incubation for 1 hour at 37°C, samples were centrifuged at 150 g for 3 minutes at room temperature (23°C). Thereafter, 150 µL of each supernatants were removed and placed in flat bottomed plates for measurement of optic density at 540 nm by “Sumal-PE2” ELISA reader (Karl Zeiss, Germany). The final APCA activity was calculated using special computer program developed in the Trakia University, and expressed as CH50 units (CH50 units correspond to 50% of complement-induced haemolysis of applied erythrocytes).

The polymorphism of C3 complement component was determined according to the method of TEISBERG [18]:

1. Buffers
   a. Stock solutions for gels - 5,5-diethylbarbituric acid sodium salt (Diemal Na, Loba-Chemie, Austria) 0.0230 M ; 5,5-diethylbarbituric acid (Reanal, Hungary) 0.0037 M ; Calcium-L(+-) lactate (Fluka AG, Switzerland) 0.0230 M ; pH=8.6.
   b. Stock solution for tray buffer (5,5-diethylbarbituric acid sodium salt (Diemal Na, Loba-Chemie, Austria) 0.061 M ; 5,5-diethylbarbituric acid (Reanal, Hungary) 0.01 M ; Calcium-L(+-) lactate (Fluka AG, Switzerland) 0.0018 M ; pH=8.6.

The first buffer was used for preparation of agarose gel (1%) where serum samples were applied. After their absorption, the electrophoresis was carried out at 20 V/cm for about 2.5 h. It was stained for 1 min with Amido Black 10 B and destained for overnight.

2. Destaining solution: ethanol, distilled water and glacial acetic acid (5:5:1) were mixed (for example, 1L destaining solution contains 450 mL ethanol, 450 mL distilled water and 100 mL glacial acetic acid). Then 2 tablespoons of active charcoal powder were added in order to allow the manifold use of the destaining solution.

3. Staining solution - 1% amido black 10 B was added to 1L destaining solution (without active charcoal ethanol),
shaked well and left for overnight. If necessary it could be filtered after a week.

**STATISTICAL ANALYSIS:**

Data were analyzed using the fixed effect MANOVA model (Program STATISTICA, StatSoft, Inc., USA). The differences were considered as significant when p values were less than 0.05.

**Results**

Prior to vaccination, lysozyme concentrations were ranged from 0.18 ± 0.04 µg/ml in the FS group to 0.24 ± 0.04 µg/ml in the SS group, but differences among genotypes were not significant. After vaccination, lysozyme concentrations were increased compared to initial (pre-vaccination) values in all groups and the percentages of increases varied from 10% for the FF group to 43% for the SS group (Table I). Again maximal values were measured in the SS and F7S genotype sheep, whereas in the FF group, the response to the vaccination was the lowest.

APCA values were the highest in the SS group and the lowest in the F7S group before the vaccination, but these variations were not significant. The vaccination induced increases of APCA values in all genotype groups except for the SS group, in which APCA values were reduced by around 5% (Table II). In the 3 other groups, the percentages of APCA increases ranged from 3% (F7S group) to 10% (FS group) (Table II). The post vaccination APCA values in the FF genotype became significantly higher than the pre vaccination APCA values in the SS genotype (p < 0.05) and consequently, maximal values were observed in the FF group whereas minimal values were encountered in the SS group.

**Discussion**

The C3 genotypes influenced the lysozyme concentrations and also the capacity of synthesis in response to a stimulus (vaccination). It was seen that the highest lysozyme concentrations were measured in the SS genotype (before and after vaccination). Moreover, in the SS genotype, lysozyme concentrations have increased in response to vaccination with the greatest intensity. In our previous study [16] we found that sheep from various breeds but belonging to transferrin genotypes TIBB, TICC and TIBC have higher lysozyme values than the other genotypes. STOYANCHEV et al. [17] reported that the broiler-chickens from B_{21}B_{21} genotype possess highest lysozyme levels. SOTIROV [14] determined that the horses from C3(3,4) genotype have also the highest lysozyme concentrations.

Vaccination has induced elevations of APCA activity particularly in the FS and FF genotypes and maximal values were obtained in the FF group. In our previous study in birds [15], it was observed that birds from the alkaline phosphatase genotype FF presented significantly higher APCA activities compared to the other alkaline phosphatase genotypes. The lowest activity was reported in birds from the SS genotype. These data were observed prior to the infection with the Marek’s disease virus. The data observed after the infection however evidenced that the highest APCA activities were obtained in the birds from the SS genotype. This strange phenomenon could be explained only if it is assumed that the birds from the SS genotype had higher possibilities for increasing the expression of genes coding for the APCA-forming components. In our case this could be regarded as a big immunobiological potential and therefore, as a bigger resistance to the virus. Phenotypically, the phenomenon is manifested as lower death rate of SS birds compared to the other genotypes.

According to WIMMERS et al. [19] the C3 gene greatly

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Lysozyme prior to vaccination</th>
<th>Lysozyme 21 days after vaccination</th>
<th>Increase (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>0.18 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>44</td>
<td>NS</td>
</tr>
<tr>
<td>FF</td>
<td>0.20 ± 0.09</td>
<td>0.22 ± 0.05</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>SS</td>
<td>0.24 ± 0.04</td>
<td>0.35 ± 0.07</td>
<td>46</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>F7S</td>
<td>0.23 ± 0.04</td>
<td>0.31 ± 0.04</td>
<td>35</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table I.** — Lysozyme concentrations (µg/ml) in dairy sheep belonging to different C3 genotypes prior to vaccination against Clostridium perfringens type C,D and Clostridium oedematiens type B and 21 days after. Results are expressed as means ± standard errors.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>APCA prior to vaccination</th>
<th>APCA 21 days after vaccination</th>
<th>Increase (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>FS</td>
<td>141.16 ± 5.91</td>
<td>154.96 ± 5.16</td>
<td>10</td>
<td>NS</td>
</tr>
<tr>
<td>FF</td>
<td>149.58 ± 8.59</td>
<td>156.70 ± 3.45</td>
<td>5</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>SS</td>
<td>150.14 ± 7.17</td>
<td>143.13 ± 4.14</td>
<td>-5</td>
<td>NS</td>
</tr>
<tr>
<td>F7S</td>
<td>139.65 ± 5.28</td>
<td>144.29 ± 5.85</td>
<td>3</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table II.** — Alternative Pathway Complement activation (APCA) (CH50) in dairy sheep belonging to different C3 genotypes prior to vaccination against Clostridium perfringens type C,D and Clostridium oedematiens type B and 21 days after. Results are expressed as means ± standard errors.
influenced the complement activity and contributed to the innate resistance against pathogenic microorganisms. In another article, WIMMERS et al. [20] revealed association of BF, HP and DRB with C3c serum concentration. The hemo-
lytic complement activity and C3c serum concentration during the experiment was affected by the interaction of DQB genotype and time of measurement. These findings promote the importance of the candidate genes for humoral mechanisms of unspcific and specific defence that provide natural resistance against many pathogens.

The vaccinations would induce some adverse effects on humoral factors of innate immunity. In some cases, the vaccinations result in severe breakdowns in immune parameters, that probably cause some observed post-vaccinal incidents. We have previously observed in 2 month-old piglets vaccinated against swine fever and erysipeloild an increased susceptibility to respiratory diseases [13]. Our hypothesis was further confirmed by the data of ABIDOV and MIRISMAILOV [1]. They observed the lowest lysozyme concentrations in humans immunized with typhoid-paratyphoid-tetanus toxoids vaccine one week after the treatment. The same authors also reported that the lysozyme concentrations were markedly reduced in men 2 weeks after antirabies vaccination. This post-vaccinal depletion of serum lysozyme could be explained by the fact that lysozyme is very active against Gram-positive bacteria (the erysipeloild causative agent belongs to this group too) [4]. On the other hand, MAZZONI and GUelli [12] and LEE-HUANG et al. [10] reported an antiviral activity of lysozyme against the rabies and HIV viruses. It is likely that its effect against the swine fever virus is identical. When pigs were vaccinated with VR2 erysipeloild vaccine, the decreased lysozyme activity was observed only with the application of the live vaccine. On the days 14 and 21, vaccinated piglets exhibited a rapid increase of lysozyme activity. These data are very similar to our experiment because the lysozyme concentrations in sheep were also enhanced on the day 21. This event is also confirmed by the reports of ABIDOV and MIRISMAILOV [1]. They immunized men and rabbits with typhoid-paratyphoid-tetanus toxoid vaccines and 3 weeks later, found that lysozyme concentrations in blood sera and saliva were increased. The results could be explained also by compensatory mechanisms of porcine and sheep organism. During the first 2 post-
vaccinal weeks, the organism fights against vaccinal antigens through a non-specific immunity (consumption of lysozyme) and at the end of this period, the synthesis of specific antibodies helps to the defence function of lysozyme. Consequently, the lysozyme, similarly to the other factors of innate immunity, is among the first factors meeting the infectious agents entering the organism and allowing it to react adequately via a specific immune response.

The alternative pathway of complement activation (APCA) is an important factor of natural immunity. It could be activated by various antigens : Gram-negative bacteria, viruses, virus-infected cells, blood parasites, inulin, agarose, sephadex, contrast media used in radiology, immune complexes of IgE and IgA [9] and therefore, it is essential for systemic defence against pathogenic agents. In contrast to lysozyme, APCA is characterized by a constantly decreased activity in pigs during the whole period of the study [13]. ANDONOVA [2] obtained data, identical to ours in pigs immunized with the VR2 vaccine. She immunized 2 month old piglets with mentioned erysipeloild vaccine and observed decreases of complement activity during the first 2 weeks and the same activity was restored after 21 days. In our study, APCA activities were generally increased by vaccination and they were strongly affected by the C3 genotype. We suppose that the effect of the vaccines is dependent on its type : monovalent and polyvalent have not so harmful effect compared to associated one [1].

In the current discussion we interpreted the changes in lysozyme and APCA independently. In fact, in living organisms, those two principal factors of the humoral immunity act jointly and simultaneously against penetrating pathogens. Evidence in this connection is provided by GLYNN [6] who reports that in the presence of specific antibodies, CPA kills Escherichia coli with a relatively long time. If lysozyme is added, this process is accelerated.

In conclusion, the vaccination against Clostridium perfringens type C,D and Clostridium oedematiens type B and the C3 genotype affiliation influenced significantly lysozyme concentrations and the alternative pathway of complement activation in sheep.

References


