HUMORAL RESPONSE AND CHANGES IN IL-2 RECEPTOR’S EXPRESSION AFTER IMMUNISATION OF PIGLETS WITH MATERNAL ANTIBODIES AGAINST AUJESZKY’S DISEASE

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Abstract

The aim of the study was the evaluation of immunological response of piglets born from sows immunised with ADV live vaccine and defining the optimal time for their active immunisation. Two weeks after the final vaccination, there were no differences in the level of serum gB antibodies among the sows and all the sows developed humoral immunity. Maternally derived antibodies (MDA) in the sera of all piglets were above the level considered to be positive until about 11 weeks of their life. Following exposure to the live ADV, a greater number of lymphocytes (including CD2+ and CD2- cells) from vaccinated animals expressed the IL-2 receptor (CD25), than those from unvaccinated ones. After virus stimulation, there were also higher expression of CD25 on CD8+ T cells in all vaccinated animals, while in non-vaccinated pigs; a decrease in such expression was evident. Based on the obtained results it might be stated that the piglet vaccination against AD at 10 and 14 weeks of life was considered to be optimal. In this age, the animals were still protected by passive immunity, but simultaneously were able to develop an active humoral response. It could be also concluded that the high level of MDA may successfully blocked the development of active immunity.

Key words: pigs, Aujeszky’s disease virus, immunisation, maternal antibodies, active immunity.

Aujeszky’s disease virus (ADV) is a swine α-herpesvirus related to the human pathogens - herpes simplex virus types 1 and 2 (HSV-1, HSV-2) (2). ADV has a board host range. It can infect most mammals except higher-order primates (2). ADV virion has a double-stranded DNA genome in an enveloped capsid capable of encoding approximately 70 proteins (7). Most of the virus-encoded proteins are glycoproteins (g), which mediated important interactions between virion and host cell. Additionally, they represent important targets for the host’s immune response. Glycoproteins gC, gE, gG, gl, and gM are designated as nonessential, i.e. deletion of the respective gene is not lethal for the virus. In contrast the absence of gB, gD, gH, gK, or gL abolished productive viral replication (7).

Some countries are free from Aujeszky’s disease (AD), while others, like Poland, are just starting an eradication program. Several authors describe that it is possible to reduce the prevalence of infection or even to eradicate the disease, using appropriate vaccines and vaccination schedules (1, 3, 15). The aim of the vaccination program is not only to create a good level of immunisation in sows but also a high and uniform level of piglets’ protection. In general, attenuated and inactivated vaccines prevent severe clinical signs and death that are often associated with the exposure of non-immune pigs to virulent ADV strain (6, 8, 15, 16). The positive aspects of passively acquired antibodies are that they provide early protection for the highly susceptible young pigs. However, it has also been shown that high titres of circulating antibodies are not solely responsible for protective immunity (5). Cell-mediated immunity is regarded as a major protective effector mechanism against virus infections, including ADV (5). Antigen specific T cell proliferation is considered as an indication of cellular immunity, although it does not provide the information on the phenotype of responding cells. To identify activated lymphocyte populations, the estimation of IL-2 receptor (CD25) expression on the surface of lymphocytes has been widely used in various species, including pigs (9, 10, 13). Lymphocytes, which have recognised their specific antigen will up-regulate their surface expression of CD25 (4). This allows binding IL 2 to its receptor thereby inducing mitosis and expansion of antigen specific clones of lymphocytes. It has been proven that up-regulation of the CD25 molecule on the surface of T cells is a useful marker of T cell antigen-specific response after in vitro stimulation in cattle (4).

The most important progress in the eradication of AD has been the elaboration of deleted vaccines allowing the differentiation between vaccinated and
wild-type virus infected animals. Most of the marker vaccines used for AD control base on the detection of gE surface glycoprotein. Moreover, the discriminatory enzyme-linked immunosorbent assays (ELISA), which detect the presence or absence of gE antibodies have been developed (7). Similarly, for the detection of antibodies in serum of vaccinated animals, the anti-gB ELISA kit was established. Based on these findings vaccination – eradication program was successfully introduced in several EU countries as well as in the USA. It consists in mass, programmed for many years’ vaccinations of reproductive and fattening pigs.

Because maternal immunity not only protects piglets against infection but it also interferes with the immunological response after vaccination, the aim of the present study was the determination of duration of passive protection transferred from sows to piglets after using live attenuated ADV vaccine and defining the optimal time for active immunisation of piglets derived from immune sows.

Material and Methods

**Animals.** Eight ADV-negative sows, France hybrids FH 900 and their litters (88 piglets), from local farm were used. The farm has closed production cycle and the basic herd consists of 50 sows. Batches of eight sows were formed every 3 weeks. Complete management and health data for the sows and their offspring were maintained. The prophylactic program at the time of pregnancy includes the vaccination of the sows with inactivated vaccines against atrophic rhinitis and colibacillosis. Production was in all-in-all out procedure with a thorough cleaning between batches. Ten days before parturition, the sows were moved to the individual farrowing pens (2×2.5 m). The piglets were weaned at approximately 28 d of their life.

**Vaccine and vaccination schedules.** Several protocols of piglet vaccination against AD were evaluated with regard to the development of immunological response in the presence of maternal derived antibodies (MDA). All the animals were vaccinated intramuscularly with a commercial vaccine Bartha strain, with guaranteed titre of $10^{6.3}$ CCID$_{50}$ in each 2.0 ml dose and oil adjuvant. Sows were vaccinated twice at 6 and 2 weeks before parturition. Piglets were assigned to six groups: five groups were vaccinated and one (group 1) served as unvaccinated control for the evaluation of the persisting of MDA in piglets. The schedule of piglets was as follows: group 2 was vaccinated following vaccine manufacturer recommendation at 10 and 14 weeks of life, groups 3 and 4 were vaccinated once at the age of 8 or 12 weeks, respectively, groups 5 and 6 were vaccinated at 7 d of age and the booster doses were administrated at 8 or 12 weeks of life, respectively. Local Ethical Commission approved all procedures involved in the study with the animals.

**Serum and colostrum samples.** Blood samples for serological examination were collected from the vena cava cranialis into serum separator tubes from all sows at the day of vaccination and 10 and 3 d before parturition, and from six randomly chosen piglets from each litters, every week in the first month of their life, and then every two weeks to the end of fattening (about 20 weeks of life). Serum was separated from the blood by centrifugation (3,000 g x 15 min).

For cytometric analyses, the samples of blood were collected to vacuum tubes, containing EDTA-K$_2$ as an anticoagulant (Medlab, Poland), two weeks after each vaccination and parallel from unvaccinated animals. The colostrum (approximately 10-15 ml) was collected from each sow at about 1 and 24 h from the beginning of parturition, using oxytocin, when necessary. Colostrum samples were centrifuged at 2,700 g for 20 min, prior freezing. Serum and colostrum samples were stored at -20°C until analysis.

**Serological test.** Specific antibodies to the gB and gE (gp1) antigens were determined using a blocking ELISA (HerdChek*Anti-PRVgB or HerdChek*Anti-PRVgp1, IDEXX Laboratories, USA), as directed by the manufacturer. Serum and colostrum samples were diluted 1:2 in a sample diluent. One hundred microlitres of diluted sample was added to wells coated with vaccine (gB) or viral (gE) antigen. The plates containing positive and negative reference and tested sera were incubated overnight in 4°C. In the next step, the plates were washed three times with a wash solution (300 µl/well) using a microplate washer (Autura 1000, Mikura Ltd., UK), and the Anti-PRV-gB or gpI:HPRO conjugate were added (100µl/well) using a microplate washer (Multiskan RC, Labsystems, Finland). Potential antigen-antibody reactions were visualised by adding 100 µl/well of 3,3’,5,5’-tetramethylbenzidine (TBM) substrate solution and incubating for 15 min. Colour reaction was stopped by adding 100 µl/well of a stop solution. Optical density (OD) was measured at 650 nm wavelength using a computerised microplate reader (Multiskan RC, Labsystems, Finland).

The presence or absence of antibodies to the examined antigen was determined by calculating the S/N ratio (OD of test serum/mean OD of negative reference serum). The samples were considered to be positive for gB antigen if S/N ratio was less or equal to 0.5, while for gE antigen if S/N ratio was lower or equal to 0.6.

**Isolation and culture of peripheral blood mononuclear cells (PBMCs).** The PBMCs were isolated from blood samples by density gradient centrifugation. The blood (3 ml) were layered on an equal volume of Histopaque 1.071 (Sigma, USA), and centrifuged for 30 min at 1,500 g at 20ºC. Buffy coat with the PBMCs was collected and washed in PBS. The isolated PBMCs were seeded in plastic vials at the density of $1 \times 10^{5}$ viable cells per ml in RPMI 1640 medium (Invitrogen, USA) with 10% of heat inactivated foetal bovine serum (PAA Laboratories GmbH, Austria) and 1% of antimycotic solution (Sigma, USA).
For immunophenotyping, the cultured PBMCs were stimulated in vitro with 50 µl of medium containing live ADV strain NIA-3 (titre 10^{3.5} TCID_{50}). In control vials, the cells were incubated without the virus.

**Phenotypic characterisation of PBMCs by two-colour flow cytometry.** For cytometric analyses, the porcine PBMCs were cultured with or without ADV for 72 h. After incubation, the cultures were centrifuged at 400 g for 10 min at room temperature, the supernatant was decanted, and the cells were washed once in PBS containing 2% of inactivated horse serum (IHS). After washing, the cells were incubated with combinations of two monoclonal antibodies (Mab) of different isotypes directed against molecules of interest. The cells were double stained for CD2/CD25 and CD8/CD25 using Mabs as follows: MSA4 directed against CD2 (T-cell antigen), 76-2-11 directed against CD8 (T cytotoxic/suppressor cell antigen), and PGBL25A directed against porcine CD25 (porcine interleukin-2 receptor) (VMRD, Pullman, USA). FITC-conjugated mouse anti-CD45 (pan-leukocyte antigen) and RPE-conjugated mouse anti-CD14 (monocyte antigen) Mabs were used together for gating the lymphocytes (Antigenix America INC, USA). Incubating the cells with an unlabeled isotype controls - mouse IgG1-UNLB (clone 15H6) and mouse IgG2a-UNLB (Clone HOPC-1) (SouthernBiotech, USA) in the place of primary antibodies, assessed nonspecific binding by labelled secondary antibodies. The cells were incubated with saturating amounts of each antibody at room temperature for 30 min. After incubation, the cells were washed twice in PBS with 2% IHS. Then, the cell suspensions were incubated for 15 min in the dark with a mixture of fluorescein isothiocyanate-conjugated rat- anti mouse IgG2a and phycoerythrin-conjugated rat- anti-mouse IgG1 (BD Pharmingen, USA). After this step, the cells were washed twice in PBS containing 2% IHS and suspended in 500 µl of buffer containing PBS and 2% of formalin. Flow cytometric analyses were performed using a Coulter Epics XL 4C flow cytometer (Beckman Coulter Company, USA).

**Statistical analysis.** Differences between means were tested for statistical significance by Student t-test using Statistica 8.0 computer program (StatSoft, Poland).

**Results**

No local or systemic adverse reactions were evidenced in all pigs after vaccination.

All the tested animals were negative to the gE antibodies. Taking into consideration the S/N ELISA ratio, four weeks after the first vaccination of sows, only one sow did not develop humoral response at the level, which could be considered as positive. The biggest differences in S/N ratio among sows were observed after the administration of a booster dose. Two weeks after the second vaccination, there were no significant differences among sows in the level of gB antibodies in serum.

In colostrum, the ELISA S/N ratio was also similar in all sows. The development of humoral immunity in sows vaccinated twice during pregnancy was shown in Fig. 1.

Maternally derived antibodies in the sera of piglets born from vaccinated sows were above level considered to be positive until about 11 weeks of their life. However, from 8-10 weeks of life, MDA S/N ratio decreased quicker than before.

An active immune response in piglets vaccinated once at 8 or 12 weeks of age, was developed only in group vaccinated at 12 weeks of age. In group vaccinated at 8 weeks of age there were no humoral response to vaccine antigen. Piglets vaccinated twice at 7 d and 8 weeks of age responded similarly as piglets from the group 3 vaccinated once at 8 weeks of life. Piglets from the group 6 had an ELISA S/N ratio considered to be positive during whole period of study, but starting from 10 weeks of life, it was lower than in the group 2. Piglets from the groups 3, 4, and 5 become negative against gB antigen from about 12 weeks of age. The decline of ELISA S/N ratio in the groups 3 and 5, and unvaccinated were similar. There were no differences in ELISA S/N ratio between piglets from the same group. Antibody responses of piglets from all groups to gE- attenuated vaccine were shown on Fig. 2.

Following the exposure to the live ADV, a higher number of cells (including CD2+ and CD2− cells) from vaccinated animals expressed the IL-2 receptor (CD25), than those from unvaccinated animals but it was statistically significant only in the group 6 (P=0.04) in comparison to non-stimulated control.

After virus stimulation, there was also higher expression of CD25 on CD8+ T cells in all vaccinated group, while in unvaccinated control a decrease in such expression was evident. In all six groups after ADV in vitro stimulation, the percentage of T lymphocytes (CD2+ cells) was higher than in non-stimulated control, but only in the groups 5 and 6, the percentage of CD2+ cells was higher than in group vaccinated according to the producer’s recommendation (group 1). Similar results were obtain for CD8+ expression, but beside unvaccinated group, a decrease in the percentage of CD8+ cells in the group 3 were also evident. In contrast to unvaccinated animals, the enhance of CD2+CD25+ cells after virus stimulation were observed in all vaccinated groups, but only in animals vaccinated twice (groups 1, 5, and 6), this increase was statistically significant (P=0.002, 0.05, and 0.01, respectively) when compared to non-stimulated control. In the groups 1 and 6, there were also significantly higher percentage of CD8+ T cells in ADV stimulated culture, than in non-stimulated one (P=0.05). In groups unvaccinated and vaccinated once (groups 3 and 4) there were no statistically significant differences within expression of all examined surface markers. Summary of the phenotypic analysis of cultured PBMCs was shown in Table 1.
Fig. 1. Antibody S/N ratio in serum and colostrum of sows.
Bold line at 0.5 ELISA S/N ratio indicates border-line between positive and negative samples. Arrows indicate moment of vaccination.

Fig. 2. Antibody responses of piglets after vaccination with live attenuated viral vaccine.
Bold line at 0.5 ELISA S/N ratio indicates border-line between positive and negative samples; d- day, w-week

Table 1
Changes in the phenotype of PBMC, cultured with or without live ADV, two week after final vaccination

<table>
<thead>
<tr>
<th>Group number</th>
<th>Positive cells (%)</th>
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<tbody>
<tr>
<td></td>
<td>CD2⁺</td>
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<tr>
<td></td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>51.1</td>
</tr>
<tr>
<td>2</td>
<td>59.8</td>
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<td>3</td>
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</tr>
<tr>
<td>5</td>
<td>49.9</td>
</tr>
<tr>
<td>6</td>
<td>57.8</td>
</tr>
</tbody>
</table>

C - PBMC cultured without ADV
V - PBMC cultured with live ADV
V/C – changes in expression of cell surface markers in cultures incubated with live ADV in comparison to non-stimulated (%)
Discussion

As was published previously by Casal et al. (3), sows vaccinated at the same time display only few differences in the level of antibodies in their serum. The results obtained in our study confirmed this finding. Two weeks after booster dose, the level of gB antibodies in serum of all sows were similar. All experimental pigs were seronegative to the gE antibodies, which indicate that they were not infected with field strain of ADV during the period of study.

The results of the presented study showed that piglets vaccinated at 1 and 12 weeks of life were better protected than those vaccinated with booster dose 4 weeks earlier. As a matter of fact, the humoral response appeared only in the group 6 and only after booster dose because the first vaccination was performed at the time when passive immunity was still high. Active immune responses in piglets vaccinated once at 8 or 12 weeks of age was remarkable only in animals vaccinated in later age. As it was mentioned, at 12 weeks of life, the passive antibodies against gB were at low titre and weaners were considered to be negative with respect to anti gB MDA. However, in next sampling periods, antibody ELISA S/N ratio was lower in animals from this group than that detected in the group 2, but still considered to be positive. Also, Vannier (14) reported that piglets vaccinated earlier (at 4 and 8 weeks of age) were not able to develop complete humoral response. Piglets vaccinated at 4 weeks of life were protected worse than those immunised 4 weeks later.

The immune response, based on ELISA S/N ratio, was the highest in group vaccinated at 10 and 14 weeks of age. So, the recommendations for the administration of vaccine given by manufacturer were in accordance with results of our study. It seems that passive immunity, whose level is relatively low at 10 weeks after birth, did not interfer already with the development of an active post-vaccinal immunity. Similar results were obtained previously by Vannier (14) and Wittman (16), who used an inactivated commercial vaccine, and by Bouma et al. (1), who used an attenuated gE 783 strain.

As the results showed, the upregulation of cells with CD25 expression was evidenced only on cells from vaccinated pigs. It may suggest that the cells were activated specifically by ADV. In the case of non-specific activation, the higher expression of CD25 surface markers should also exist on PBMCs from unvaccinated group. The expression of CD25 after in vitro stimulation of PBMCs from challenged animals with different immunological history was also investigated by Suradhat et al. (13). The mentioned authors did not find significant differences in the number of cells expressing CD25, regardless to immunological history of tested pigs. Similar observations were made in our study, because we did not find a correlation between numbers and time of vaccinations and extent of the upregulation of the expression of CD25 markers on PBMCs from different groups of pigs.

The most enhance of CD25 expression after in vitro stimulation of PBMCs with the virus was evidenced on the cells carried the CD2 phenotype (T lymphocytes). On CD2 cells (including B-cells and γδT cells) an increase in the activation marker expression were lower (up to 10%) and no antigen-specific, because it was observed in unvaccinated groups also. We noted a greater expression of CD25 on CD8+ T cells after virus stimulation in all vaccinated group, while in group unvaccinated, a decrease in such expression was observed.

As it was demonstrated, the enhance of CD8 T cells proliferation and CD25 expression plays a critical role in the protective immune response against virus infected cells and maintenance of immunological memory (12, 13). In addition, recent studies suggest that antigen-activated CD8 T lymphocytes can eliminate or control viral infection by secretion of antiviral cytokines, such as γ-interferon and tumour necrosis factor-α (11).

Our results have shown that an active immunisation of piglets born from sows immunised with live attenuated ADV vaccine could be successful when maternal antibody levels are about 0.35 ELISA S/N ratio. The mentioned value was observed in unvaccinated group about 9-10 weeks of life. Above data indicate that at the moment of vaccination, animals are still protected by passive immunity, but simultaneously are able to develop an active immunity. It could be also concluded that the high level of MDA may successfully block the developmental of active immunity. Therefore, there is a need for careful consideration of the best moment for the piglets’ vaccination. Optimally, the moment of vaccination should be connected with evaluation of serological profile of particular herd.

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References