EFFECT OF INTRANASAL PI3V AND BRSV ADMINISTRATION ON THE ALTERATIONS IN SELECTED PERIPHERAL BLOOD LEUKOCYTE SUBPOPULATIONS IN CALVES

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Received for publication September 7, 2009

Abstract

The aim of the study was to investigate the influence of two components of Rispoval 3 vaccine i.e. PI3V and BRSV administered to calves by the intranasal route on the changes of in selected subpopulations of peripheral blood leukocytes. Eighteen clinically healthy calves were divided into two equal groups. In calves from the experimental group both live attenuated viruses i.e. parainfluenza virus (PI3V) and bovine respiratory syncytial virus (BRSV) were intranasally administered into the animals. Directly before administration, the lyophilised form of the viruses were suspended in 10 ml of the sterile physiological saline. Then each experimental animal was intranasally treated with 2 ml of the suspension, while the control calves received a similar value of placebo, which was a sterile physiological saline. The blood samples were collected at 6, 24, 48, 72, and 168 h after the administration. The following parameters were assayed: the percentage of CD2+ (T-lymphocytes), CD4+ (T-helper lymphocytes), CD8+ (T-suppressor/cytotoxic lymphocytes), and WC4+ (B-lymphocytes) cells, and also leukocytes expressing CD11b+ (α sub-unit of β2 integrin). The obtained results indicated a statistically-significant (P<0.05) increase in all the examined parameters in the experimental group of animals when compared with the controls. Intranasal PI3V and BRSV administration in calves considerably stimulated both cellular and humoral specific components of immune response in the affected animals. It probably indicates effective protection against infection as a result of applied immunisation.

Key words: calves, leukocytes, PI3V, BRSV.

Bovine respiratory syncytial virus (BRSV) is a negative-stranded RNA virus belonging to Paramyxoviridae family (5, 25). BRSV is responsible for respiratory disease in cattle with seroprevalence of 30%-70% (1, 9, 14). Isolates of the virus has have been received from cattle descended from different parts of the world, i.e. Europe, America, and Asia (15, 20, 23). Young cattle less than one year old are mainly susceptible to BRSV infection (14, 17, 26) and development of severe clinical signs (17, 26, 31). Independently, the virus causes acute respiratory failure in affected calves. The symptomatology of BRSV infection is biphasic (21). Disease symptoms mainly come down to clinical signs for respiratory system parts, i.e. cough, nasal discharge, polypnea, and abdominal dyspnoea (30).

Parainfluenza type 3 virus (PI3V) derives from the same family as BRSV (29). However, PI3V infection usually has a subclinical or mild course (10). The role of PI3V in diseases of the respiratory system in cattle is still important considering the predisposition to secondary infections (27).

BRSV and PI3V belong to factors involved in the pathogenesis of bovine respiratory disease (BRD). BRD is responsible for wide economic losses in the population of calves and young cattle. Bovine herpesvirus 1 (BHV1) is also a consequential factor of viral origin in BRD. Viruses constitute a primary source of disease in BRD, as opposed to secondary bacterial infections i.e. Mannheimia haemolytica, Pasteurella multocida or Histophilus somni, and mycoplasmal agents inter alia Mycoplasma bovis (3). Besides, viruses facilitate colonisation and multiplication of bacteria as a result of changes in surface proteins of epithelium cells in the respiratory system, ciliary apparatus damage, and increase of mucus viscosity (21).

Rispoval 3 vaccine (Pfizer Animal Health S.A.) is composed of modified live viruses i.e. BRSV and PI3V in lyophilised form as well as inactivated bovine viral diarrhoea virus (BVDV) type 1 as suspension. The vaccine stimulates immunological system of treated calves. The duration of postvaccinal immunity averages 6 months with reference to BRSV and BVDV (type 1). The immunity against PI3V is unknown. Effective immunoprophylaxis of BRD has a high significance in
stock-raising because it may consequently contribute to a decrease in calf mortality, an improvement in the clinical status of the animal, and in the productivity rate i.e. weight gain or reduction of veterinary costs (antibiotic use etc.). However, till now, the most manufactured commercial polyvalent viral vaccines against BRD were only recommended for intramuscular administration. Practically, little is known about the immunological effect of vaccines or their components applied by the intranasal route with regard to cellular immune response in young cattle. Deficiency in this representative data determines the intensive development of the study, particularly using an advanced technique of analysis such as flow cytometry. The application of this technique is of great significance, especially with reference to the evaluation of the immunological status of animals, on the basis of following parameters, e.g. lymphocyte subpopulations, i.e. CD2+, CD4+, CD8+, γδ TCR+ , WC1+ T cells, WC4+, CD21+CD32+ B cells, and NKp46+ NK cells (2, 6, 16), β2-integrin, and its cellular adhesion molecule (CAM) - α2-integrin (CD49d), the main factors of neutrophil migration (24), the expression of interleukin-2 receptor α-chain (IL-2Rα) (18) and others. CD45 and CD45RO expression (19), virus specific proliferation, interferon-γ response (34), the surface expression of CD25 on CD4+, CD8+ and γδ T-cells and, additionally, the presence of virus-neutralising antibodies and virus-specific IgG were the theme of researches concerning the cell- and humoral-mediated immune response to BRSV (22). In reference to PI3V infection, virus specific serum antibody responses were examined (29).

Therefore, the aim of the present study was to investigate the influence of two components of Rispoval 3 vaccine i.e. PI3V and BRSV administered to calves by the intranasal route on the changes of subpopulations of peripheral blood leukocytes.

**Material and Methods**

**Animals.** Eighteen clinically-healthy, Black and White Lowland breed calves, aged 6-8 weeks, were divided into two equal groups. Experimental procedures and animal management protocols were undertaken in accordance with the requirements of the Local Ethics Committee on Animal Experimentation of the University of Life Sciences in Lublin, Poland.

**Study protocol and sample collection.** In the experimental calves (group I), the two live attenuated viruses i.e. PI3V and BRSV were administered intranasally into the animals. Directly before the application, the lyophilised form of the viruses was suspended in 10 ml of the sterile physiological saline. Then each experimental animal was intranasally treated with 2 ml of the suspension, while the control calves (group II) received the similar value of placebo in the form of sterile physiological saline. The blood samples were collected into tubes containing K3EDTA as the anticoagulant (0.07 mol/mL of blood) at 6, 24, 48, 72, and 168 h after the administration.

**Blood analysis.** The following parameters were assayed: the percentage of CD2+ (T-lymphocytes), CD4+ (T-helper lymphocytes), CD8+ (T-suppressor/cytotoxic lymphocytes), and WC4+ (B-lymphocytes) cells, and, additionally, leukocytes-expressing CD11b+ surface marker, i.e. a so-called α sub-unit of β2 integrin.

**Immunophenotyping of bovine peripheral blood lymphocytes.** The immunophenotyping of peripheral blood lymphocytes expressing CD2 (T-cell antigen), CD4 (T-helper antigen), CD8 (T-cytotoxic/suppressor cell antigen), WC4 (bovine B-cell antigen), and CD11b surface marker was performed by the use of Epic XL 4C Flow Cytometer (Beckman-Coulter Company, USA). A panel of monoclonal antibodies (MCAs) directed against bovine leukocyte cluster of differentiation antigens (CD) was used to differentiate peripheral blood leukocyte subpopulations. It comprised those which recognise CD45 (MCA832F mouse anti-bovine CD45:FITC, clone number-CC1), CD14 (MCA156C cross-reacting mouse anti-human CD4:RPE-Cy5, clone number - TuK4), CD2 (MCA833F mouse anti-bovine CD2:FITC, clone number - CC42), CD4 (MCA1653F mouse anti-bovine CD4:FITC, clone number - CC8), CD8 (MCA837F mouse anti-bovine CD8:FITC, clone number - CC63), CD11b (MCA1425 mouse anti-bovine CD11b) and WC4 (MCA1648 mouse anti-bovine WC4, clone number - CC55). It was bound additionally by the secondary F(ab’2) rabbit anti-mouse immunoglobulin conjugated to FITC- STAR9B). All these monoclonal antibodies were manufactured by Serotec Ltd (UK). Immunofluorescent analysis of peripheral blood leukocytes was performed according to Beckman-Coulter Operator’s Guide Procedure. FITC-conjugated anti-CD45 and RPE-Cy5-conjugated anti-CD4 MCAs were used together for gating the lymphocytes. The analysis of suitable surface marker expression was done directly from whole blood basing on OptiLyse Immunotech preparation standard procedure. Fifty microliters of whole blood were incubated at room temperature for 15 min with suitable monoclonal antibodies. Then 250 µl of lysing solution (OptiLyse C, Immunotech) was added to all the blood samples and incubated again under the same conditions. After red blood cells lysis, leukocytes were washed with PBS containing 5% foetal calf serum and resuspended in 500 µl of PBS with foetal calf serum. The cell suspension was analysed, using a flow cytometer and a logarithmic amplifier. SYSTEM II 3.0 software for the Cytometer was used to the data acquisition (listmodes) and their cytometric analysis (histograms). Additionally, alive Multigraph programme was used to calculate and display the data.

**Statistical analysis.** The statistical significance of differences between the mean values recorded in the experimental groups of animals and the controls was compared using Student’s t-test at P<0.05.
**Results**

The results of immunophenotyping of selected peripheral blood leukocyte subsets are summarised in Table 1. Immunophenotyping showed higher values in the percentage of CD2⁺ (T lymphocytes), CD4⁺ (T helper lymphocytes), CD8⁺ (T suppressor/cytotoxic lymphocytes), and CD11b⁺ (α sub-unit of B2 integrin) in calves after intranasal PI3V and BRSV administration in comparison to the controls. The rise in these parameters was observed during the whole experiment. The percentage of CD4-positive cells in the experimental animals was significantly higher than in the controls after 24 h up to 168 h of the experiment and mean values fluctuated between 32.17% and 30.62%. In group I, the percentage of CD2⁺ lymphocytes was significantly higher in comparison with the controls from 6 h of the experiment and ranged from 65.9% to 65.03%. In the control group, these mean values ranged between 52.43% and 56.25%. At the same time, the percentage of CD8⁺, WC4⁺, and CD11b⁺ significantly increased too. The differences between both examined groups of calves were most marked in the case of WC4⁺. The highest value of WC4⁺ was observed after 6 and 24 h of the experiment and mean values fluctuated between 36.03% and 35.75%, respectively. CD4:CD8 positive T-cell ratio in peripheral blood of calves was lower during the whole study in the experimental animals when compared to the controls. Statistically-significant values were observed at 6 and 48 h of the experiment (Fig. 1).

**Table 1**
The immunophenotyping of selected peripheral blood leukocyte subpopulations in calves intranasally vaccinated with BRS and PI3 viruses (group I), and unvaccinated (group II)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Time of observation (h)</th>
<th>CD2⁺ (%)</th>
<th>CD4⁺ (%)</th>
<th>CD8⁺ (%)</th>
<th>WC4⁺ (%)</th>
<th>CD11b⁺ (%)</th>
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<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
<td>Group II</td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td>57.28 ± 1.60</td>
<td>54.60 ± 1.30</td>
<td>25.12 ± 1.13</td>
<td>24.62 ± 1.13</td>
<td>15.78 ± 0.90</td>
<td>15.53 ± 0.85</td>
</tr>
<tr>
<td>6</td>
<td>65.90 ± 4.14*</td>
<td>52.43 ± 1.61</td>
<td>27.63 ± 2.94</td>
<td>25.95 ± 0.87</td>
<td>21.85 ± 1.85*</td>
<td>15.28 ± 1.51</td>
</tr>
<tr>
<td>24</td>
<td>68.97 ± 2.23*</td>
<td>53.87 ± 1.19</td>
<td>32.17 ± 1.08*</td>
<td>22.55 ± 1.36</td>
<td>22.05 ± 1.85*</td>
<td>22.55 ± 2.30</td>
</tr>
<tr>
<td>48</td>
<td>67.65 ± 2.75*</td>
<td>55.32 ± 1.88</td>
<td>29.77 ± 2.70</td>
<td>26.47 ± 1.10</td>
<td>20.28 ± 1.47*</td>
<td>15.47 ± 1.85</td>
</tr>
<tr>
<td>72</td>
<td>67.48 ± 2.17*</td>
<td>56.37 ± 2.22</td>
<td>31.58 ± 2.77*</td>
<td>23.68 ± 2.43</td>
<td>21.82 ± 3.55*</td>
<td>14.78 ± 1.64</td>
</tr>
<tr>
<td>168</td>
<td>65.03 ± 4.83*</td>
<td>56.25 ± 1.77</td>
<td>30.62 ± 2.84*</td>
<td>24.92 ± 1.38</td>
<td>19.47 ± 1.42*</td>
<td>14.47 ± 1.27</td>
</tr>
</tbody>
</table>

* P<0.05

**Fig. 1.** The CD4:CD8 positive T-cell ratio in peripheral blood of calves intranasally vaccinated with BRS and PI3 viruses (group I), and unvaccinated (group II) * P<0.05.
Discussion

The intranasal administration of PI3 and BRS viruses was applied in the experimental calves at the age of 6-8 weeks. These animals did not have a fully formed mature active immunity against all infections. Maternal antibodies may be maintained in calves for up to 6 months of their life (21). It is worth noticing that mucosal route of vaccination seems to be more resistant to immunosuppressive effect of maternal antibodies when compared with the parenteral route (4). In the experiment, two live attenuated viruses were used in the vaccination of calves. Modified-live virus (MLV) vaccines are responsible for the activation of both cellular and humoral immunological mechanisms (21).

Besides, this kind of vaccine causes more effective stimulation of production virus neutralising (VN) and fusion-inhibiting antibodies (8) but less with reference to the virus-specific (non-neutralising) IgG (7, 8, 32). On the other hand, at present it is difficult to say what is the role of the different ways (e.g. subcutaneous, intranasal) of vaccine application for the protection efficacy and active immunity level. There are differences between MLV and inactivated vaccines, also with reference to T helper lymphocyte response. Since MLV vaccines induce T helper 1 (Th-1) lymphocytes as opposed to the inactivated ones that are responsible for the stimulation of Th 2-type response (12, 33, 34). Th1 lymphocytes synthesise cytokines i.e. interleukin-2 and interferon-γ that intensify the function of cytotoxic T lymphocytes and play a role in the activation of macrophages (13).

Immunophenotyping analysis of leukocytes subsets in the peripheral blood of calves in the study indicated a significant (P<0.05) increase in CD4+ cells (T-helper lymphocytes) in the experimental calves until 168 h after vaccination when compared with the controls. Therefore, this rise showed the mobilisation of the cellular immune response in the affected calves. The vaccination of calves caused an increase in the WC4% percentage, a substantial marker of bovine B lymphocytes. This rise in B-cell response in treated animals indicated a probable induction of Th-2 lymphocytes that participate in humoral immunological mechanisms, i.e. increase of CD25+ and proliferation of in B lymphocytes and production of immunoglobulins as a consequence. This activity of Th-2 cells takes place by the agency of interleukin-4 (IL-4), IL-5, IL-6, and IL-10 (13). Apart from WC4%, CD11b+ is present at surface of B lymphocytes in cattle, and this last marker first of all on neutrophils. In these animals CD11b+ has higher affinity to peripheral blood in comparison with lymph nodes (35). The rise of cells expressing CD11b+ in the experimental group of calves was observed. This rise additionally indicates the distinct activation of B-cell response following the vaccination of the animals.

One of the major components of BRSV nucleocapsid is nucleoprotein (28), which is responsible for viral transcription and replication (36). This protein is recognised by BRSV-specific bovine CD8+ (11). The significant (P<0.05) increase in the number of these cells observed in the experimental group of calves probably indicates the mobilisation of T-suppressor/cytotoxic lymphocytes in response to the administration of graft virus. That rise is important considering the function of T-s/c cells and its role (cytotoxic T lymphocytes) in convalescence and resistance to reinfection (21).

The determination of the CD4:CD8 ratio plays an important role in the evaluation of conditions, i.e. a vaccination or infections affecting the immune system of animals (2). A lower CD4:CD8 positive T-cell ratio was observed in the peripheral blood of the experimental calves during the whole study when compared to the controls. It resulted from increased values of both CD4+ and CD8+ T cells in vaccinated animals but to T-s/c the lymphocyte’s advantage. This advantage indicates the particular importance of this kind of immune response in the generation of cell-mediated immunity against infection as a result of applied vaccination.

The increase of T-cell response in the experimental group following vaccination found confirmation in other studies. The immunisation of calves with the use of MLV vaccine caused the rise of T-cell response i.e. CD4+, CD8+, and γδ T-cells and additionally antibodies (virus neutralising antibodies and virus-specific IgG) (22).

The increase in the CD2+ percentage (T lymphocytes) following the vaccination of calves probably resulted not only from the elevation of both CD4+ and CD8+ responses but also the rise of surface markers of other cells of lymphatic system, i.e. killer, natural killer, and lymphokine-activated killer. The significance of the cells in the mechanisms of organism defense is substantial considering their strong cytotoxic activity (35).

In conclusion, intranasal PI3V and BRSV administration in calves considerably stimulated both cellular and humoral specific components of immune response in the affected animals, manifested by a significant increase in the investigated subsets of peripheral blood leukocytes. It can probably determinate the effective protection against infection as a result of applied immunisation.

References

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