EVALUATION OF PERIPHERAL BLOOD LEUKOCYTE SUBPOPULATIONS BY FLOW CYTOMETRY IN CALVES TREATED WITH MANNHEIMIA HAEMOLYTICA LEUKOTOXIN

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Abstract

The aim of the study was to evaluate the effect of Mannheimia haemolytica leukotoxin (Lkt) on cellular immune response in clinically-healthy calves given intravenously 25 µg per animal of M. haemolytica A1 Lkt. The alternations of peripheral blood leukocytes were examined with a flow cytometry. The Lkt treated calves were compared with the non-treated controls before (0) and 1, 2, 3, 4, 5, 6, and 24 h after the treatment. The following parameters were assayed: white blood cell (WBC) count, percentage of polymorphonuclear leukocytes (PMNL), mid-size leukocytes, total percentage of lymphocytes and their subsets: CD2+ (T lymphocytes), CD4+ (T helper lymphocytes), and CD8+ (T suppressor/cytotoxic lymphocytes) with CD4+: CD8+ ratio, and also WC4+ cells (B lymphocytes). The obtained results showed that in the treated calves, the WBC count, the percentages of PMNL, mid-size leukocytes, and some subpopulations of peripheral lymphocytes (CD2+, CD4+, CD8+) were significantly lower compared with the controls during the first of three hours of the experiment, and then the parameters increased and returned to the physiological level.

Key words: cattle, Mannheimia haemolytica leukotoxin, leukocytes, flow cytometry.

Leukotoxin (Lkt) has been established as the major virulence factor in Mannheimia (Pasteurella) haemolytica, which is one of the most important aetiopathogenic agents of bovine respiratory disease (BRD). The aetiology of BRD is multifactorial where viral (BHV, BVD, BRSV, PI-3), mycoplasmal (M. bovis, M. bovirhinis, Ureaplasma diversum), and bacterial (M. haemolytica, H. somni) agents, as well as the environmental factors (inappropriate livestock management like mistakes in animal nutrition, transport, handling, veterinary interventions etc.) are also significant. Therefore, the effective control of BRD is very difficult. At present, BRD is a great economic problem in cattle husbandry, especially in young and feedlot animals all over the world. It accounts for approximately 30% of the total cattle deaths worldwide and is associated with an annual economic loss of over one billion dollars in North America alone (1). On the other hand, in Poland, the estimated cost concerning only deaths and elimination of cachexia affected animals accounts for about 10 million Euro per year. It is evident that the BRD problem is very important, and searching for new aspects of its etiopathogenesis, particularly with regard to M. haemolytica virulent factors, is certainly necessary for the development of the more effective BRD control programmes.

M. haemolytica produces many other potentially virulent factors (7, 14). Among them leukotoxin (Lkt) is a specific to bovine leukocytes exotoxin produced by all known serotypes and several unclassified strains of M. haemolytica during the rapid logarithmic phase of growth of the bacterium. Despite the fact that the all serotypes secrete Lkt, the leukotoxin isoform produced by M. haemolytica biotype A, serotype 1, has the most visible cytotoxic properties in relation to bovine leukocytes. Therefore, the name of this exotoxin is connected with its specific toxic properties directed to ruminant leukocytes. It has been discovered that bovine leukocytes exposed to low doses of the exotoxin show reduced phagocytic and killing activity with regard to engulfed bacteria. On the other hand, higher concentration of the agent causes complete destruction of the leukocytes leading to their swelling and bursting (4). Many potentially profitable reactive substances (e.g. free radicals, lisosomal enzymes, proteases) in relation to phagocytes (neutrophils,
monocytes) are released from the destroyed cells and then they stimulate different pathological lesions in host affected lung tissues. The process manifests itself in an acute lobar fibrinonecrotising pneumonia. This histopathological picture is characteristic of BRD. All the changes are consequences of Lkt action and the development of lung inflammatory cascade regulated additionally by some pro-inflammatory cytokines. However, in this destroying cascade process the initiation role of Lkt is the most important and crucial. It also influences other mechanisms in a host organism, both concerning typical inflammatory reactions and non-specific immune response. The last-mentioned dependences are not well known, and therefore they require further complex studies in the future.

The purpose of the present study is to evaluate the effect of *Mannheimia haemolytica* Lkt on cellular immune response in the experimentally Lkt treated healthy calves, with regard to the differentiation of bovine peripheral blood leukocyte subpopulations.

### Material and Methods

#### Animals. The study was performed on 20 clinically-healthy, Black and White Lowland breed calves, aged 6-8 weeks, and with an average body weight of 78 ±3.1 kg. Experimental procedures and animal management protocols were undertaken in accordance with the requirements of the Animal Care and Ethics Committee.

#### Study protocol and sample collection. The calves were fed traditional ruminant feeding stuffs (C-J feed concentrate, hay, water *ad libitum*). After one week of acclimatisation, the calves were randomly divided into two equal groups. The experimental animals (group I) received intravenously 25 µg per animal of *Mannheimia haemolytica* A1 Lkt. The Lkt was obtained according to the procedure described by Clinkenbeard et al. (3), modified by the use of RPMI 1640 medium without supplements (13). It was administered in the native form. The remaining calves served as controls (group II) and received 0.9% NaCl in the same route and comparable volume as Lkt.

For immunological examinations, blood was collected from all the animals at the same time in the morning by jugular vein puncture just before (0 sample; 0 time point) and at 1, 2, 3, 4, 5, 6, and 24 h after Lkt administration. The blood samples were collected into tubes containing K₃EDTA as the anticoagulant (0.07 mol/mL of blood).

#### Blood analysis. The following leukocyte indices were determined: white blood cell (WBC) count and their leukogram, *i.e.* the percentage of polymorphonuclear leukocytes (PMNL; neutrophils), lymphocytes (LYM), and mid-size leukocytes (MID – the total values of all peripheral blood monocytes, eosinophils, and basophils). The examinations were performed using an automatic haematological analyser (Swelab AC 960 Counter).

#### Immunophenotyping of lymphocytes. The immunophenotyping of lymphocytes expressing CD2 (T-cell antigen), CD4 (T-helper antigen), and CD8 (T-cytotoxic/suppressor cell antigen) with their relative proportions (CD4:CD8 ratio) and WC4 (bovine B-cell antigen) surface marker was performed by the use of Epics XL 4C Flow Cytometer (Beckman-Coulter Company, USA). A panel of monoclonal antibodies (MCAs, Serotec Ltd, UK) directed against bovine leukocyte cluster of differentiation antigens (CD) was used to differentiate peripheral blood leukocyte subpopulations. It comprised MCAs recognising CD45 (MCA832F mouse anti-bovine CD45:FITC, clone number-CC1), CD14 (MCA156C cross-reacting mouse anti-human CD14: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), and WC4 (MCA1648 mouse anti-bovine WC4, clone number - CC55) bound additionally by the secondary F(ab')2 rabbit anti-mouse immunoglobulin FITC conjugated to FITC- STAR9B). Immunofluorescent analysis of the leukocytes was performed according to the Beckman-Coulter Operator's Guide Procedure. FITC-conjugated anti-CD45 and RPE-Cy5-conjugated anti-CD14 MCAs were used together for gating the lymphocytes. The analysis of a suitable surface-marker expression was done directly from whole blood based on the OptiLyse Immunotech preparation standard procedure. Fifty micro litres of whole blood were incubated at room temperature for 15 min with appropriate 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 erythrocyte lysis, leukocytes were washed with PBS containing 5% foetal calf serum and then 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, a Multigraph program was used to calculate and display the data.

#### Statistical analysis. The statistical significance of differences between the mean values recorded in experimental and control animals was compared using Student’s *t*-test at *P*<0.05, *P*<0.01, and *P*<0.001.

### Results

The results of routine peripheral blood leukocyte analysis (WBC, PMNL, MID, LYM) and lymphocyte immunophenotyping (CD2, CD4, CD8, WC4-positive cells) are summarised in Tables 1-2. The obtained results show that significant differences in WBC, PMNL, and MID were already observed at the 1⁶ h of the experiment in group I (Table 1). However, among these indices, the WBC mean values were significantly lower for the longest time, *i.e.* until the 6⁰ h of the study in experimental calves, if compared with
Instead, looking at the lowest significant mean values of the parameters noted in group I, there were 1.9 ±0.9 x 10^9/L of WBC at the 3rd h after the Lkt administration (P<0.001), 18.6 ±1.1% of PMNL at the 2nd h (P<0.05), and 4.9 ±0.9% of MID at the 3rd h too (P<0.01). In control animals, the same indices were 9.4 ±1.9x10^9/L, 23.2 ±2.8%, and 6.9 ±1.0%, respectively at the same time points as above. Generally, from the 3rd h of the study the mean values of these parameters were increasing significantly up to the 24th h after the Lkt administration, and finally there were the physiological values for the calves aged 6-8 weeks. However, in this time the total number of WBC (11.6 ±1.8x10^9/L) was higher in the treated animals as compared with its initial value (8.8 ±1.5x10^9/L) and the controls (9.9 ±2.2x10^9/L).

The immunophenotyping of peripheral blood lymphocytes of calves showed the visible lower values for the percentage of CD2 (T lymphocytes), CD4 (T helper lymphocytes), and CD8-positive cells (T suppressor/cytotoxic lymphocytes) in the animals treated with the leukotoxin (Table 2). In these experimental calves the percentage of T lymphocytes was significantly lower (P<0.05) in comparison with the controls from the 1st to 3rd h after the Lkt administration and ranged from 53.4% to 55.7%. By contrast, these values were between 66.4% and 68.1% in the controls at the same time of the observation. The changes of T lymphocyte subpopulations had a similar nature and manifested significantly lower values between the 1st and 3rd h of the study compared with those recorded in the control group. The percentage of CD4-positive cells trended downwards from 28.7% (0 point) to 21.7% at the 3rd h of the observation and then gradually increased, while in the controls the mean values fluctuated between 29.2% and 30.5%. A similar tendency regarding the percentage of CD8-positive cells was also observed. During the first of 3rd h of the study, the values decreased significantly from 23.3% to 18.6%. In the same time, the percentage of CD8-positive cells in the controls changed imperceptibly and remained within the stable range from 24.4% to 25.2%. The changes presented above reflected those noted in the relative-proportion evaluation of CD4-CD8 positive cells (Fig. 1). The ratio was significantly higher (P<0.05) in experimental animals at the first hour of the study (1.45 ±0.09) as compared both with the control (1.23 ±0.04) and its initial value (1.23 ±0.03).

In contrast with the results presented above, the percentage of B lymphocytes (WC4+) was comparable in both groups of calves; however, its values were distinctly higher in the experimental animals during the first three hours of the study. In both groups the mean values of this parameter were not statistically significant and ranged between 20.4% and 31.2%.

### Table 1

White blood-cell count and leukogram in of the peripheral blood of calves treated with *M. haemolytica* Lkt (Group I), and control (Group II)

<table>
<thead>
<tr>
<th>Time of observation (h)</th>
<th>Parameter</th>
<th>WBC x10^9/L</th>
<th>PMNL%</th>
<th>MID%</th>
<th>LYM%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
</tr>
<tr>
<td>0</td>
<td>8.8 ±1.5</td>
<td>9.9 ±2.2</td>
<td>23.4 ±1.7</td>
<td>24.3 ±3.1</td>
<td>7.2 ±1.1</td>
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<td>1</td>
<td>2.7 ±1.9a</td>
<td>9.1 ±1.1</td>
<td>19.7 ±2.1a</td>
<td>24.0 ±2.4</td>
<td>5.8 ±0.8a</td>
</tr>
<tr>
<td>2</td>
<td>2.0 ±0.6b</td>
<td>9.7 ±2.3</td>
<td>18.6 ±1.1b</td>
<td>23.2 ±2.8</td>
<td>5.7 ±0.9b</td>
</tr>
<tr>
<td>3</td>
<td>1.9 ±0.9a</td>
<td>9.4 ±1.9</td>
<td>18.9 ±1.2a</td>
<td>22.9 ±1.0</td>
<td>4.9 ±0.9a</td>
</tr>
<tr>
<td>4</td>
<td>2.6 ±1.3b</td>
<td>8.9 ±1.8</td>
<td>20.7 ±3.2b</td>
<td>22.1 ±2.1</td>
<td>5.9 ±0.8b</td>
</tr>
<tr>
<td>5</td>
<td>3.1 ±1.9a</td>
<td>9.0 ±2.2</td>
<td>22.8 ±3.0a</td>
<td>23.3 ±3.1</td>
<td>6.9 ±1.8</td>
</tr>
<tr>
<td>6</td>
<td>4.5 ±1.6a</td>
<td>8.9 ±2.0</td>
<td>22.9 ±2.0a</td>
<td>24.1 ±3.9</td>
<td>7.1 ±1.5</td>
</tr>
<tr>
<td>24</td>
<td>11.6 ±2.1</td>
<td>9.9 ±2.2</td>
<td>26.7 ±3.2</td>
<td>24.9 ±2.0</td>
<td>7.7 ±1.1</td>
</tr>
</tbody>
</table>

a P<0.05; b P<0.01; c P<0.001

### Table 2

Immunophenotyping of peripheral blood lymphocytes in calves treated with *M. haemolytica* Lkt (Group I), and control (Group II)

<table>
<thead>
<tr>
<th>Time of observation (h)</th>
<th>Parameter</th>
<th>CD2%</th>
<th>CD4%</th>
<th>CD8%</th>
<th>WC4%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
<td>Group II</td>
<td>Group I</td>
</tr>
<tr>
<td>0</td>
<td>66.4 ±3.7</td>
<td>68.8 ±2.8</td>
<td>28.7 ±1.3</td>
<td>28.5 ±3.6</td>
<td>23.3 ±3.1</td>
</tr>
<tr>
<td>1</td>
<td>55.7 ±5.6a</td>
<td>68.1 ±2.7</td>
<td>24.9 ±2.2a</td>
<td>30.4 ±1.7</td>
<td>17.2 ±2.8a</td>
</tr>
<tr>
<td>2</td>
<td>54.3 ±4.0a</td>
<td>67.5 ±4.3</td>
<td>22.6 ±2.5a</td>
<td>29.3 ±3.9</td>
<td>19.4 ±2.1a</td>
</tr>
<tr>
<td>3</td>
<td>53.4 ±3.3a</td>
<td>66.4 ±2.0</td>
<td>21.7 ±2.3a</td>
<td>29.2 ±1.0</td>
<td>18.6 ±2.2a</td>
</tr>
<tr>
<td>4</td>
<td>59.2 ±3.8</td>
<td>64.6 ±3.2</td>
<td>25.3 ±2.5</td>
<td>27.3 ±2.5</td>
<td>19.7 ±3.0</td>
</tr>
<tr>
<td>5</td>
<td>60.3 ±4.3</td>
<td>65.3 ±2.2</td>
<td>27.1 ±1.3</td>
<td>30.2 ±3.2</td>
<td>20.1 ±2.2</td>
</tr>
<tr>
<td>6</td>
<td>62.6 ±2.8</td>
<td>67.2 ±2.6</td>
<td>28.5 ±1.9</td>
<td>30.5 ±2.0</td>
<td>21.5 ±1.1</td>
</tr>
<tr>
<td>24</td>
<td>64.3 ±2.9</td>
<td>67.9 ±1.7</td>
<td>27.9 ±1.2</td>
<td>30.0 ±2.6</td>
<td>22.1 ±2.6</td>
</tr>
</tbody>
</table>

a P<0.05; b P<0.01; c P<0.001
Discussion

In the present study, there was observed both the significant decrease in total WBC (leukopenia) and their individual leukocyte subsets in blood of calves treated with *M. haemolytica* Lkt. In particular, the PMNL, MID, and T lymphocyte decrease was noted. Among T lymphocytes, the most visible effect was related to CD4 and CD8-positive cells. Visible changes were also observed regarding PMNLs and MID cells, especially during the first three hours of the study. This special susceptibility of bovine PMNLs, monocytes, (one of the most important components of MID cell subpopulation) and T lymphocytes to Lkt-induced cytalysis, manifested a significant decrease in the cells observed in our study, and probably resulted in the presence and expression of all known three β2 integrins (CD11a/CD18; CD11b/CD18, CD11c/CD18) serving as receptors of *M. haemolytica* Lkt (2, 5, 6, 9, 10). The Lkt also binds to non-ruminant leukocytes, but does not induce cytosis (8). Moreover, during the binding process by the specific leukocyte adhesions molecules, signal-inducing trans-membrane pore formation is generated, leading to efflux of K\(^+\), influx of Ca\(^2+\), colloidal osmotic swelling, and leukocyte cytosis (4). This destructive mechanism leads to a distinct decrease in the peripheral blood leukocyte number and leukopenia. The leukopenia resulted from the toxic influence of *M. haemolytica* A1 Lkt, demonstrating species-specific effect with respect to bovine leukocytes (1, 2, 11). Similar results were also shown in another study of cattle infected with *M. haemolytica* (12).

The generally-decreasing tendency of the total number of peripheral blood leukocytes found in our study in the experimental calves was also correlated with the alterations in individual lymphocyte subpopulations in these animals. The percentage of T (CD2\(^+\)) lymphocyte and its subsets, i.e. CD4\(^+\) (Th) and CD8\(^+\) (Ts/c), distinctly decreased in the calves during the study (Table 2). Their differences were statistically significant as compared with the control animals (Group II) at the first, second, and third hour of the study. Moreover, in the affected animals, the CD4:CD8 ratio increased significantly during the first hour after the Lkt administration (Fig. 1). This increase in treated calves was mainly due to the rise in the proportion of CD4\(^+\) cells and the decline in the proportion of CD8\(^+\) cells. The ratio of T-lymphocytes that express the CD4 antigen to those that express the CD8 antigen is a very important immune diagnostic measurement. Consequently, this value is commonly assessed in the diagnosis and staging of diseases affecting the immune system, including both viral and bacterial infections. The CD4:CD8 imbalance, together with diminished lymphoproliferative capacity, may lead to a weaker T cytotoxic-mediated immunity and increased susceptibility to infectious diseases in cattle and especially in young cattle. Our study also emphasises the importance of this parameter for immune-response evaluation in calves affected by *M. haemolytica* leukotoxin.

On the other hand, concerning WC4\(^+\) results, the distinct increase in its mean values was observed in experimental animals (Group I) from 20.4 ±1.6% to 31.2 ±3.6% during the first three hours of the study. However, these values were not significant as compared with the controls, and at the end of the experiment, they decreased and were similar to those recorded in the control group (Table 2). The results came out of target-cell response, i.e. the activated lymphocyte subsets presented above, to the Lkt presence in blood of affected animals. The spectacular changes in the calves treated with *M. haemolytica* Lkt, observed as lymphopenia, are evidence of the recirculation of T (CD2\(^+\)) lymphocyte

![Fig. 1. CD4:CD8 positive T cell ratio in peripheral blood of calves treated with *M. haemolytica* Lkt (Group I) and control (Group II)](image)
subpopulations to lymphoid organs in order to activate them. It has crucial implications, because the activated Th (CD4+) lymphocytes play an important role in cellular (cytotoxicity of lymphocytes and macrophage activation) and humoral (a stimulating effect on the increase and differentiation of B lymphocytes, and finally the immunoglobulin synthesis) immunity. Moreover, Ts/c (CD8+) lymphocytes are responsible for the regulation of the T lymphocyte function and their antibody-dependent cytotoxicity (ADC), whereas bovine B (WC4+) lymphocytes produce immunoglobulins as an essential part of specific defence mechanisms in the affected host organism.

From the results obtained in our study, the conclusion can be drawn that the single intravenous administration of M. haemolytica A1 leukotoxin (Lkt) to clinically healthy calves caused the occurrence of the transient leukopenia manifested in the significant decrease in total peripheral WBC count, the percentages of PMNL, MID cells, and the some lymphocyte subpopulations (CD2+, CD4+, CD8+).

References