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RELATIONSHIP BETWEEN BLOOD LYMPHOCYTE PHENOTYPE, DRB1 (MHC CLASS II) GENE POLYMORPHISM AND SOMATIC CELL COUNT IN EWE MILK

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Abstract

The aim of the study was to analyse the variability of the leukocyte population and lymphocyte subpopulation in peripheral blood in ewes of different age and mammary gland health status and with different genotype in DBR1 (MHC II) gene. Blood samples were collected 3 times from 24 ewes of Polish Heath Sheep at the age of 6, 12 and 24 months. Milk samples were collected from ewes at the age of 24 months, during their first lactation period. The status of the mammary gland was established according to the number of somatic cell count (SCC) in 1 ml of milk, with 300 000 cells/ml established as a maximum for a healthy gland. Consequently, all the ewes were divided into two groups with cell count <300 000 (healthy = H) and > 300 000 (mastitic = M). A statistically significant increase in number of neutrophils, eosinophils, monocytes, CD2+ and CD4+ lymphocytes and statistically significant decrease in the number of CD19+, WC1-N2+ and CD8+ lymphocytes and MHC II+ cells in older animals (24 months of age) were observed. Additionally, 24 month old ewes of the H group being at the age of 6 months showed higher percentage of CD2+, CD19+ ($P \leq 0.05$), CD8+ ($P \leq 0.05$) lymphocytes as well as MHC II+ cells ($P \leq 0.01$) comparing to the ewes of the same age from M group. Also not significant increase in CD4+ and WC1-N2+ lymphocyte number was found. Analysis of microsatellite polymorphism of DRB1 gene fragment identified the presence of 8 alleles. One of them, of 526 bp length, was of particular interest as it was most frequently found in group H ewes. Moreover, its presence was associated with several times higher percentage of CD2+, CD8+, CD19+ lymphocytes and MHC II+ cells in peripheral blood, when compared to other alleles.

Key words: ewes, age, mammary gland, leukocytes, phenotypes, genes, polymorphism.

Health status of mammary glands is considered an important economic factor in sheep breeding, as it does not only influence quantity and quality of milk produced, but also numbers of lambs successfully reared. The number of somatic cells in 1 ml of milk is a basis of mammary gland health status evaluation (2, 24). Somatic cell count remains therefore one of the selection criteria in dairy cattle breeding programmes in several countries (17, 18). Because of an insufficient progress in mastitis control with traditional veterinary methods, more consideration is now put into more natural approach, based on animal natural immunity against inflammation of the mammary gland. Numerous studies on immune response against different infections showed that most of the immune system components change significantly with age (7). T-lymphocytes remain major controlling cells of the immune system with their main functions being mediation with cytokines, produced after antigen-stimulated activation of cell surface molecules. Immune response against bacterial infections depends primarily on recognition of bacterial antigens and their presentation by MHC (major histocompatibility complex) molecules to T-lymphocytes (20).

The aim of this study was to describe the variability of leukocyte population and lymphocyte subpopulation in peripheral blood according to the age and mammary gland health status in sheep and microsatellite polymorphism in DBR1 (MHC II) gene, encoding the β chain of DR molecule.

Material and Methods

The study was performed on 24 Polish Heath Sheep ewes, randomly selected from the flock bred at the Agricultural Station in Żelazna. Blood samples were collected from the cervical external vein in the 6^{th}, 12^{th}, and 24^{th} month of age. At the last blood collection, milk samples were also collected in the 4^{th} week of the first lactation, from each half of the udder. The inbreeding coefficient in the studied group was 0.027.

Cytometric analysis was conducted by applying specific monoclonal antibodies against CD2, CD4, CD8, CD19, WC1-N2 and MHC II (VMDR Inc. Pullman) and double staining procedure using fluorescein isothiocyanate (FITC) and R-phycoerythrine (PE) conjugated anti-mouse IgG or IgM (Medac) (4, 12, 22). Red cells were lysed by adding FACS Lysing Solution (Becton-Dickinson). Results were read with flow cytometer FACStrak (Becton-Dickinson) and data controlling SimulSET and CellQuest programmes.

Health status of the mammary gland was assessed on the basis of somatic cell count (SCC) in 1 ml of milk, counted with Somacount 150-Bantley analyser, with borderline established at 300 000 cells/ml (3). Any result above that level was qualified to M (mastitic) group, whereas that below the borderline qualified to H (healthy) group.

DNA for DRB1 gene polymorphism study was isolated from peripheral blood according to PC (phenolo-chlorophorm method) and analysis of the length of DRB1 (exon2/intron2) microsatellite sequence was performed using PCR method (9). The length of the obtained PCR products was established with POP4 capillary sequencer and Gene Scan 2.1 (Perkin Elmer) programme.

The obtained results were analysed statistically with variance analysis (SPSS 11.5 PL Windows). In order to standardise distribution, all results of cytometric analysis were transformed into logarithmic scale (8, 20).

Results

Results of peripheral blood cytometric analysis in ewes of different age groups are shown in Table 1. The highest percentage of peripheral blood lymphocytes was found in sheep at 12 months of age and the lowest at 24 months. Conversely, neutrophil and monocyte counts were the lowest. Eosinophil count tended to increase with the age.

When lymphocyte subpopulations are taken into considerations, only age-related increase in the number of CD2+ and CD4+ ($P<0.01$) lymphocytes was found. In other lymphocyte subpopulations their significant age-related decrease was observed. CD4/CD8 ratio was increasing significantly with the age.

Cytological studies showed marked differences in SCC, varying from 15 000 to 4 365 000 among all samples. However, it did not exceed 300 000 in half of the animals examined. The peripheral blood cells' variability in relation to the age and mammary gland status is presented in Table 2.

When comparing adult (24 month) ewes of H and M groups, lowered percentage of neutrophils ($P<0.01$), eosinophils, MHC II+ cells, B (CD19+) and WC1-N2+ lymphocytes ($P<0.01$) in H group, with simultaneous increase in percentage of monocytes and total lymphocyte ($P<0.01$) and, specifically, T lymphocytes CD2+, CD4+, and CD8+ were found. Adult ewes of H group, when examined at the age of 6 months, showed increased percentage of CD2+, CD19+ ($P<0.05$), CD8+ ($P<0.05$) and MHC II+ cells ($P<0.01$) and some, yet not significant, increase in CD4+ and WC1-N2+ lymphocytes. Additionally, differentiation in T lymphocyte percentage was reflected in calculated CD4:CD8 ratio that showed reversed proportions of both lymphocyte type (group H – 0.65, group M – 1.61). Similar relations (except for CD4+ increase) were found in ewes at 12 months of age. The percentage of lymphocyte subpopulations in each group, as shown in Table 2, deviated distinctly from mean percentage calculated for given age group (Table 1).

Microsatellite polymorphism analysis showed the presence of 8 alleles in DRB1 locus (Table 3). The highest frequency was established for the alleles of 488 bp (37.5%), and 508 bp, 516 bp, 526 bp (15 – 17.5%) length. Those frequencies were compared with cytological analysis of milk. The highest SCC was found in ewes carrying most frequently 488 bp and 508 bp alleles (383 000 and 407 000, respectively), whereas the lowest was observed in ewes that carried 516 bp and 526 bp alleles with the highest frequency (98 000 and 77 000, respectively).

Table 1

Variability in total leukocyte count and lymphocyte subpopulations in peripheral blood in relation to ewe age

Cells	Age (months)		
	6	12	24
Leukocytes (%)			
Lymphocytes	47.85 ^{aA}	56.35 ^{aB}	36.50 ^{AB}
Neutrophils	46.44 ^{c}	36.99 ^{cC}	52.15 ^{C}
Eosinophils	2.58 ^{DE}	4.42 ^{dD}	7.28 ^{dE}
Monocytes	2.46 ^{F}	1.53 ^{FG}	2.74 ^{G}
Lymphocyte subpopulations (%)			
CD2+	52.55	51.83	55.50
CD19+	34.90 ^{d}	28.56	26.15 ^{d}
WC1-N2+	8.33 ^{e}	8.00	5.42 ^{e}
CD4+	19.55 ^{AB}	26.00 ^{AC}	35.55 ^{BC}
CD8+	24.53	24.05	18.92
MHC II+	48.70	41.52	41.05
CD4:CD8	1.12 ^{A}	1.15 ^{B}	1.90 ^{AB}

a, c, d, e - $P \leq 0.05$; A, B, C, D, E, F, G – $P \leq 0.01$.

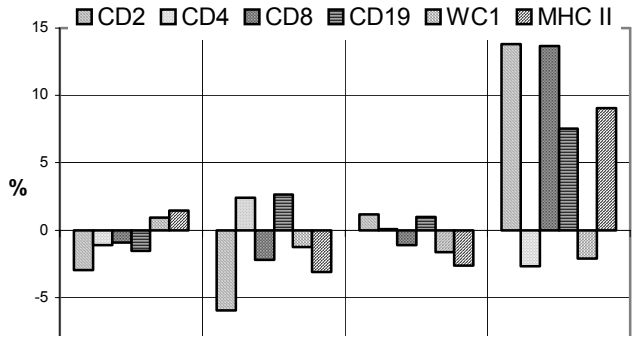
Table 2
Variability in total leukocyte count and lymphocyte subpopulations in peripheral blood in relation to age and udder health status of ewes aged 24 months

Cells	Age (months)					
	6		12		24	
	H	M	H	M	H	M
Leukocytes (%)						
Lymphocytes	44.85	50.84	57.04	55.60	46.11^{A}	28.,64^{A}
Neutrophils	49.91	42.96	35.48	38.66	43.67^{A}	59.10^{A}
Eosinophils	2.42	2.75	4.89	3.96	6.04	8.47
Monocytes	2.11	2.86	1.63	1.43	3.00	2.55
Lymphocyte subpopulations (%)						
CD2+	57.80	47.30	55.00	48.36	59.78	52.00
CD19+	40.30^{a}	29.50^{a}	29.50	27.55	23.78	28.10
WC1-N2+	6.53	10.69	7.28	8.88	4.84	6.00
CD4+	18.10	21.00	26.41	25.55	36.44	34.81
CD8+	34.65^{a}	17.26^{a}	27.69^{a}	20.58^{a}	19.29	18.71
MHC II+	58.50^{A}	38.90^{A}	43.33	39.55	38.55	43.1
CD4:CD8	0.65^{a}	1.61^{a}	0.97	1.35	1.93	1.88

a - P ≤ 0.05; A - P ≤ 0.01; H - SCC < 300 000; M - SCC > 300 000.

Table 3
Frequencies of DRB1 (MHC II) alleles and mean SCC in milk of ewes carrying a specific allele

Allele	Frequency		SCC
Length (bp)	n	%	median
488	15	37.5	383
508	6	15.0	407
516	7	17.5	98
520	1	2.5	602
526	6	15.0	77
530	1	2.5	1025
540	1	2.5	108
566	3	7.5	61
Total	40	100.0	-



allele (bp)	**488**	**508**	**516**	**526**
CD2	-2.98	-5.97	1.19	13.82
CD4	-1.13	2.41	0.09	-2.68
CD8	-0.93	-2.21	-1.10	13.67
CD19	-1.52	2.65	1.00	7.55
WC1-N2	0.91	-1.23	-1.66	-2.13
MHC II	1.44	-3.10	-2.62	9.06

Fig. 1. Variability in subpopulation lymphocyte count in peripheral blood in lambs at 6 months of age presented as a difference between mean lymphocyte count in the carriers of a specific allele and mean lymphocyte count in the carriers of the remaining alleles of DRB1 gene.

The presence of 526 bp allele in a sheep genotype was related to the multiple increase in CD2+, CD8+, CD19+ lymphocytes and MHC II+ cells in peripheral blood when compared to their counts in the presence of other alleles.

Discussion

The results obtained indicate that the age of an animal has significant influence on the variability of lymphocyte subpopulation. The cytometric studies revealed the age-related increase in the number of CD2+ and CD4+ lymphocytes with simultaneous decrease in CD8+, CD19+ and WC1-N2+ lymphocytes. Similar age-related changes in immune cells were reported in numerous studies, both in animals (25, 26) and humans (10, 19). These results are, nevertheless, difficult to compare, as obtained from specimens of different age, coming from totally different populations.

In each group of sheep the variability of peripheral blood leukocytes in 24 month old ewes was noted in connection to those at 6 and 12 months of age. Such comparison enables to conclude whether the different health status can be attributed to the percentage of respective immune system cells, counted at the age of 6 and 12 months.

The increase in SCC may result from bacterial infection in the mammary gland. Such infections lead to immune response in which the recognition of pathogen antigens and their presentation to the competent immune cells remain the crucial process. MHC particles, T-lymphocytes and B-lymphocytes are involved in the process. The role of both CD4+ and CD8+ lymphocytes in generating immune response is well-established. CD4+ product appears to be an important "cell-to-cell interaction" molecule required for cognate interactions between T - and B-lymphocytes that are needed to generate an antigen specific response (13). The CD8+ lymphocyte is thought to have the same function as CD4+, mainly in cell-to-cell adhesion and signal transduction. The result obtained in this study revealed the age-related increase in CD4+ number. In CD8+ count, however, a slight decrease was noticed. CD4+:CD8+ cell ratio is an important marker for the maturation of the immune system (5). In our study we found age-related increase in this ratio, with its additional divergence linked to udder health status. The significant age-related increase in this ratio was observed in ewes with SCC that was below the established 300 000/ml at the age of 24 months (i.e. H group). Our results suggest that changes in percentage of immune system cells during animal growth and development may influence it adult immunity. The main role in the development of the immunity is attributed to CD8+ and CD19+ lymphocytes. Their numbers in peripheral blood in healthy ewes at the age of 6 and 12 months were significantly higher than those in group classified as mastitic. The dominance of CD8+ lymphocytes was previously described mainly in ruminant mammary gland secretions (14, 16, 23).

Considering the significant role of MHC molecules in immune response generation, we analysed polymorphism in microsatellite sequence length of DRB1 (exon 2/intron 2) as well as its possible relation to SCC in milk. It is evident that the antigen binding groove, encoded by the second exon of the gene, has a high rate of polymorphism and this determines the capability of MHC products to bind to a wide range of peptides. The close relation is also known to exist between the length of the microsatellite sequency we studied and exon 2 restriction polymorphism (1). We found high frequencies for four alleles: 488 bp, 506 bp, 516 bp, and 526 bp. Among them the most frequent allele present in healthy ewes was that of 526 bp and it was also linked to the highest percentage of CD2+, CD8+ and CD19+ lymphocytes as well as MHC II+ cells. It can be, therefore, suggested that the presence of 526 bp allele may be a useful marker of ovine resistance to mastitis.

Suggestions concerning the possible use of MHC genes/antigens as genetic markers of either resistance or susceptibility to mastitis had been previously put forward by several authors (20, 15). Dietz *et al*. (6) used milk SCC as a measure of udder health and found that the BoLA class II DRB3 allele DRB3-2*16 (identified using a PCR-RFLP method) may play a role as a risk factor for higher SCC. Some other studies (11) also confirm potential use of DRB3.2 alleles (DRB3-2*8 and DRB3-2*16) as markers of mastitis susceptibility.

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