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# Immune Repertoire of Sheep Blood B-Cells in the Postvaccination Immune Response

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**Abstract**—The main specific functions of B-cells are carried out with the help of membrane receptors. Binding of the receptor to its ligand activates a cascade of reactions leading to the formation of memory cells and protective antibodies. The study was conducted to analyze the phenotypic profile of sheep blood B-cells in the process of postvaccination immune response to an inactivated vaccine against pathogens of acute intestinal diseases of animals. Romanov sheep at the age of 1.5 years were immunized twice with a 2-week interval. Studies were conducted before vaccination and on the seventh, 14th, and 21 days of the immune response. The phenotype of B-cells was determined by immunoperoxidase staining using monoclonal antibodies to CD receptors. A simple radial immunodiffusion reaction was used to assess IgG levels. Vaccination of sheep with an inactivated vaccine caused an increase in the level of the general population of leukocytes and a subpopulation of B2 cells ($p < 0.05$). The absolute number of leukocytes increased on the seventh and 14th days of the primary immune response and on the seventh day of the secondary immune response compared to the initial values. The number of B2 lymphocytes with the CD5 – $IgM^+$ phenotype increased and amounted to $9.0 \times 10^6$ cells/mL on day 7 and $11.2 \times 10^6$ cells/mL on day 14 of the primary immune response ($4.5 \times 10^6$ cells/mL before the introduction of the vaccine). An increase in the level of the CD5 – CD19 + IgM + CD20 + lymphocyte subpopulation was noted in the first 2 weeks of the immune response and was 2.5 times higher than the initial value. Data were obtained on the absence of the effect of vaccination on the level of lymphocytes with the CD5 + CD19 + IgM + phenotype (B1-cells), which did not change during either the primary or secondary immune response, which indicates the independence of priming of the two main subpopulations of B-cells. The structural components of the immune system in the process of immunogenesis are not activated simultaneously, and when evaluating the effectiveness of vaccination, functional interrelations of immunological indicators are of particular importance. A strong correlation between the indicators of B2-cells and the level of total immunoglobulins of class G ($r = 0.9$) indicates a positive effect of vaccination.

**Keywords:** B-cells, postvaccination immune response, CD markers, immunoperoxidase staining

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## INTRODUCTION

The study of B-cell activation, which is directly related to the receptor profile of cell membranes, in the process of the immune response is extremely important not only for understanding the physiological foundations of immunity but also for creating new ones as well as testing of existing vaccine preparations. Experimental data on membrane-dependent reactions of antigen presentation is a necessary basis for the development of modern vaccine-manufacturing technologies. Their efficiency directly depends on the adequacy of the processes of proliferation and differentiation of B-lymphocyte subpopulations, which can be determined by the expression of CD-receptors. Their level can serve as a definite marker of the stages of the formation of a postvaccination immune response.

Each stage of B-lymphocyte differentiation corresponds to a certain immune repertoire of CD-markers: pro-BI-cell to CD19, pro-BII to CD19CD20, pre-BI to CD19CD20mPreBCR, and pre-BII to cIgMCD19CD20CD21. The next stage is the formation of the B-cell antigen receptor (BCR), during which a monomeric $sIgM^{lo}$ molecule corresponding to its cytoplasmic form (cIgM) appears on the membrane. Mature naive B-lymphocytes are characterized by the $sIgM^{hi+}$ IgDCD19CD20CD21 phenotype.

There are three subpopulations of B-lymphocytes in cattle—B1a $CD5^+$, B1b $CD5^-$, and B2—which have different functional properties. The membrane receptor CD5 allows us to distinguish between subpopulations: $CD19^+$ $CD5^+$ B1-cells and $CD19^+$ $CD5^-$ B2-cells [1].

B1aCD5$^+$ lymphocytes are designed to respond quickly to the most common antigens of the bacterial cell walls in the barrier cavities.

Natural antibodies secreted by B1a lymphocytes (IgM and IgA) are predominantly specific for thymus-independent antigens. CD5$^+$ B-cell levels can be elevated in autoimmune diseases.

It is also known that lymphocytes expressing the CD5$^+$ molecule are a target for bovine leukemia virus [2].

The CD19 marker protein belongs to the immunoglobulin superfamily. Many of these proteins are different polymers in which homologous Ig-structures of different chains interact with each other. Each such structure is encoded by a separate exon. The CD19 B-cell receptor plays an important role in positive signal transduction in both T-dependent and T-independent immune responses. CD19 serves as a marker protein for B-cells from the earliest stage of their differentiation. Another marker receptor for B lymphocytes is the CD20 molecule, which is expressed starting from proB-cells but is absent on plasma cells. The function of CD20 in the formation of $Ca^{2+}$ channels and in the regulation of the activity of B-cells, including antibody genesis, is known.

The CD20 molecule, moving into lipid rafts, coordinates the work of receptor proteins [3]. For maturation, B-cells require not only the CD19 and CD20 molecules but also the regulator of complement activation CD21. On the surface of lymphocytes, it appears only at the pre-B-cell stage. Activation occurs when CD21 binds to opsonins C3bi (an inactivated form of the complement component C3b) on the cell surface, which is accompanied by phosphorylation of the CD19 membrane protein. Simultaneously with the expression of CD21, the IgM μ-chain appears in the cytoplasm of the pre-B-cell. After the completion of the gene rearrangement, the B-cell expresses the complete immunoglobulin molecules on its surface that serve as the basis for the B-cell receptor (BCR). The function of BCR is to bind antigen and conduct a signal inside the cell for further differentiation and proliferation of the lymphocyte. The cytoplasmic part of the membrane sIgM consists of three amino acid residues, which are insufficient for the formation of structural motifs; therefore, the signal is transmitted through the associated Igα and Igβ glycoproteins, which are phosphorylated. Membrane monomers IgM are oriented by the Fab-region towards the external environment, while the Fc-fragment is in contact with the cell surface. S-IgM contains—at the C-terminus of heavy chains—domains formed by hydrophobic amino acids, which hold the Ig molecule on the outer surface of the membrane [4].

The most numerous population consists of B2 lymphocytes, which differentiate into plasma cells and produce all known immunoglobulin isotypes with a huge variety of antigenic determinants. Under the influence of the antigen, B2-lymphocytes begin to divide intensively, immunoglobulins of the IgG class—IgA and IgE less often—appear on the cell surface instead of IgM and IgD characteristic of naive B-cells, and the frequency of mutations increases [5].

This causes the formation of numerous new receptor variants of B-lymphocyte subclones. If mutations lead to a weakening of the affinity of the receptor for the antigen and the B-cells do not receive supporting signals from the antigen-fixing dendritic cells, they undergo apoptosis. In the case of an increase in the affinity of the receptor for the antigen, the cells survive and leave the follicle, migrate to the lymph nodes, spleen, and also (especially with a secondary response) to the bone marrow, where they differentiate into plasma cells and secrete antibodies. Soluble forms of B-cell membrane immunoglobulins, namely, homologues of antigen-binding sites of the sIgM receptor of B-cells, serve as antibodies to various antigenic determinants [6].

It is already clear today that many phenotypically different cell populations are present in the blood. Using the methods of immunocytochemistry, it is possible to search in the peripheral blood for the most characteristic marker phenotypes of cells for a certain stage of immunogenesis, which, from our point of view, is advisable to use to assess the effectiveness of vaccines. However, despite the development of modern technologies and methodologies, as the situation with the coronavirus pandemic shows, many problems still remain unresolved. Therefore, a detailed study of the body's cellular responses to various types of pathogens is of paramount importance for the development of infectious immunology.

The aim of the research is to determine the phenotypic profile of B-cells in the peripheral blood of sheep during the postvaccination immune response to the associated inactivated vaccine.

## MATERIALS AND METHODS

The studies were carried out before the introduction of the vaccine, then on the seventh, 14th, and 21st days of the postvaccination immune response. An associated, inactivated vaccine against colibacillosis, salmonellosis, klebsiellosis, and proteinaceous infection (Vaccine OKZ, Agrovet) was administered to healthy sheep of the Romanov breed at the age of 1.5 years (five animals) twice according to the manufacturer's instructions. The animals were kept in accordance with GOST 33215–2014. Blood lymphocytes were isolated by Histopaque-1077 density gradient centrifugation at 3000 rpm within 45 minutes. The concentration of mononuclear cells in the suspension was adjusted to 1.0 ... 0.5 × $10^6$ cells/mL.

The number of B-cells in the blood was determined by the method of indirect immunoperoxidase staining (IPO) [7]. To remove exogenous immunoglobulins, 100 μL of a suspension of mononuclear blood cells was

**Table 1.** CD repertoire of bovine B-lymphocytes derived from blood during the immune response ($M \pm m$)

| Indicator | Initial value | Day of postvaccination immune response | | |
|---|---|---|---|---|
| | | 7 | 14 | 21 |
| Leukocytes thous./μL | 7.0 ± 1.2 | 16.8 ± 3.6* | 20.0 ± 4.3* | 10.0 ± 1.5 |
| $CD5^+$: | | | | |
| %[1] | 39.0 ± 8.4 | 12.0 ± 9.0 | 14.0 ± 6.7 | 32.5 ± 3.1 |
| $10^6$/mL | 2.73 ± 0.5 | 2.4 ± 1.3 | 2.8 ± 1.0 | 3.2 ± 2.1 |
| $CD19^+$: | | | | |
| % | 48.4 ± 11.3 | 51.6 ± 16.4 | 33.7 ± 8.9 | 55.0 ± 15.0 |
| $10^6$/mL | 3.4 ± 1.0 | 8.8 ± 4.9* | 6.6 ± 2.9* | 5.5 ± 0.5 |
| $CD20^+$: | | | | |
| % | 52.5 ± 7.7 | 34.0 ± 8.9 | 47.5 ± 12.5 | 50.0 ± 9.0 |
| $10^6$/mL | 3.7 ± 1.3 | 5.8 ± 1.8 | 9.6 ± 1.8* | 5.0 ± 1.3 |
| $CD21^+$: | | | | |
| % | 60.0 ± 3.2 | no data | 37.4 ± 2.0 | 40.0 ± 6.4 |
| $10^6$/mL | 4.2 ± 0.7 | | 7.4 ± 1.9 | 4.0 ± 0.5 |
| $sIgM^+$: | | | | |
| % | 64.0 ± 5.1 | 53.4 ± 12.1 | 56.3 ± 9.4 | 60.0 ± 3.7 |
| $10^6$/mL | 4.5 ± 0.9 | 9.0 ± 3.9* | 11.2 ± 3.2* | 6.0 ± 1.0 |

[1] %, from sheep blood lymphocytes; $10^6$/mL, absolute cell count at $10^6$/mL;
* differences from the initial value are significant at $p < 0.05$.

treated with 1% citric acid solution for 1 min and centrifuged five times in phosphate buffer (pH 7.2) at 1100 rpm for 5 min. The cell suspension was fixed with ethanol on a glass slide. Peroxidase blockade was performed with a 0.3% hydrogen peroxide solution for 10 min; 1% BCA (pH 7.2 ... 7.4) was used as a blocking solution.

Cells were incubated for 60 min at room temperature and thoroughly washed. Monoclonal antibodies to cattle sIgM and to CD5,19,20,21 markers of human B-cells (Sorbent) were then added to the fixed cells, and they were incubated for 60 min at room temperature in a humid chamber. A goat antimouse IgG peroxidase-conjugate was used as secondary antibodies. A 3-amino-9-ethylcarbazole staining kit (AEC Staining Kit, Sigma) was used to visualize the peroxidase.

AEC-positive cells were identified by red-brown staining when viewed under a microscope (×1000). Total immunoglobulins of class G in the blood serum of sheep were determined by the method of simple radial immunodiffusion.

## RESULTS AND DISCUSSION

The effectiveness of the immune response to a vaccine directly depends on chemical reactions involving surface receptors of the lymphocyte. One of the main mechanisms of the postvaccination immune response is genesis of antibodies, the stages of which can be determined by the CD-profile of B-cells.

The results of the conducted studies indicate that the number of leukocytes significantly increases, compared with the background values, on the seventh and 14th days of the primary immune response and decreases on the seventh day of the secondary immune response (see Table 1).

On the seventh day after the introduction of inactivated microbial cells of the *E. coli* 09:K99, *E. coli* 0138:K88, *S. dublin*, *S. enteritidis*, *S. typhimurium*, *Kl. pneumoniae*, *Pr. vulgaris*, and *Pr. mirabilis* vaccine strains, B-cells with the $CD19^+$ $sIgM^+$ phenotype prevailed in the blood of the vaccinated animals. An increase in the number of $CD5^-CD19^+sIgM^+CD20^+$ lymphocytes was noted from the seventh day, and their number was 2.5 times greater by the 14th day of immunogenesis than before the vaccine administration. The number of lymphocytes with the $CD5^+CD19^+sIgM^+$ phenotype (B1 cells) in the postvaccination immune response did not change.

CD21 expression is characteristic of immature and naive B-cells and decreases after cellular activation. Thus, on the seventh day of the secondary immune response, the relative number of $CD21^+$ B-lymphocytes decreased by 1.5 times.

On the first day of the postvaccination immune response, the number of $CD5^-CD19^+sIgM^+$ cells,

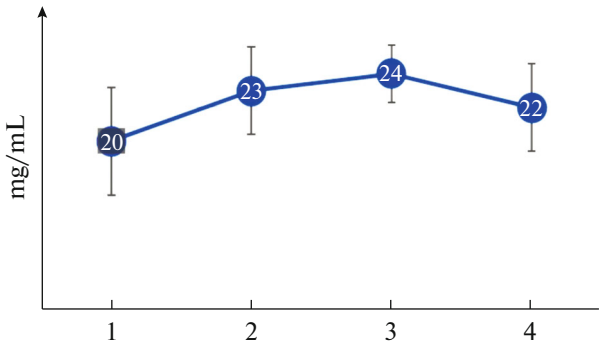


**Fig. 1.** Level of IgG in the blood serum of sheep during the immune response: 1—before vaccination; 2—seventh; 3—14th; 4—21st day of postvaccination immune response.

which are then transformed into plasma cells and memory B-cells, which are crucial for the long-term protective effect of vaccines, through mutations of immunoglobulin genes and isotype switching, increased from $1.8 \times 10^6$ up to $6.6 \times 10^6$ cells/mL ($p < 0.05$).

Differentiation of B-lymphocytes into cells secreting IgG antibodies is accompanied by intense mutagenesis, mainly in the dark zones of the germinal centers at the centroblast stage [8]. The reaction in the embryonic center of the spleen lasts about 20 days before the appearance of clones with a higher affinity for the antigen than the original cells. Memory cells do not differ morphologically from naive B-lymphocytes, but the CD repertoire changes.

It is known that memory B-cells are characterized by low and high affinity for the antigen. Insufficient affinity of BCR for the antigen ensures, on the first day of the immune response, the maintenance of the polyreactivity of memory B-cells, which determines a wider range of interactions with various pathogens [9]. The ingress of a certain antigen into the body causes the formation of memory cells not only to this antigen but also to other pathogens. A subpopulation of memory B-cells with the $CD19^+sIgM^+$ phenotype, similar to naive B lymphocytes, can recognize many antigens, but with a faster response to them. An increase in the level of B-cells with the $CD5^-CD19^+sIgM^+CD20^+CD21^-$ phenotype on the 14th day after vaccination by 2.5 times ($p < 0.05$) may indirectly indicate the presence of memory B-cells with low affinity in the blood. It should be noted that, the expression density of receptors on the cell membrane of B-lympocytes was low ($CD^{lo}$) during the study period of the postvaccination immune response.

When studying humoral immunity, a tendency to an increase in the concentration of polyspecific class G immunoglobulins in the blood serum of vaccinated sheep from 20.0 mg/mL (initial value) to 23.0 mg/mL on the seventh day and 24.0 mg/mL by 14 day of the immune response (see Fig. 1) was revealed. The switch of lymphocytes from IgM production to the synthesis of immunoglobulins of other isotypes occurs as a result of the recombination of repeated switching sites and deletion of intermediate $C_H$-genes.

An increase in the level of total serum IgG during the postvaccination immune response correlates with an increase in the number of $CD19^+$ – ($r = 0.8$), $CD20^+$ – ($r = 0.89$), and $sIgM^+$ cells ($r = 0.95$). Such associations indicate the inclusion of a subpopulation of B2 lymphocytes in the process of differentiation with the formation of plasma cells synthesizing specific antibodies of class G. In turn, low correlation coefficients ($r = -0.1$) between the parameters of $CD5^+$ cells and IgG content confirm the independence of priming of B-cell subpopulations.

Thus, current data on the role of various B-cell subpopulations in the postvaccination immune response indicate the relevance of this line of research. Understanding how different pathogens induce and modulate immune cells is essential for the development of vaccine formulations. The phenotypic diversity of B-lymphocytes allows us to study the mechanisms of their proliferation and differentiation in order to search for marker indicators of an effective immune response. As a result of the studies, a functional relationship between the parameters of B2 cells and the level of total class G immunoglobulins was established. During the primary and secondary postvaccination immune response, an increase in the expression of B-cell receptors CD19, CD20, and sIgM was shown against the background of their low density on the cell membrane. The number of B1-cells after vaccination did not change and was comparable to the initial value.

It should be noted that the indicators of polyspecific IgG and CD repertoire of primed B-cells during the primary immune response were higher than the same parameters after the administration of a booster dose of the vaccine. In general, the results of studies indicate a positive effect of vaccination on genesis of antibodies and are consistent with modern knowledge of the mechanisms of the immune response formation.

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## COMPLIANCE WITH ETHICAL STANDARDS

*Conflict of interest.* The authors declare that they have no conflicts of interest.

*Statement on the welfare of animals.* All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

## REFERENCES


1. Pape, K.A., Maul, R.W., Dileepan, T., et al., Naive B cells with high-avidity germline-encoded antigen receptors produce persistent $IgM^+$ and transient $IgG^+$ memory B cells, *Immunity*, 2018, vol. 48, no. 6, pp. 1135–1143. https://doi.org/10.1016/j.immuni.2018.04.019
2. Ezdakova, I.Yu., Kapustina, O.V., Gulyukin, M.I., et al., Characterization of B1-cells during experimental leukomogenesis, *Vopr. Virusol.*, 2020, vol. 65, no. 1, pp. 35–40. https://doi.org/10.36233/0507-4088-2020-65-1-35-40
3. Mesin, L., Ersching, J., and Victora, G.D., Germinal center B cell dynamics, *Immunity*, 2016, vol. 45, no. 3, pp. 471–482. https://doi.org/10.1016/j.immuni.2016.09.001
4. Ezdakova, I.Yu., *Retseptory immunnogo uznavaniya u zhivotnykh* (Immune Recognition Receptors in Animals), Moscow: Sputnik+, 2008.
5. Weller, S., Braun, M.C., Tan, B.K., et al., Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire, *Blood*, 2004, vol. 104, no. 12, pp. 3647–3654. https://doi.org/10.1182/blood-2004-01-0346
6. Kruetzmann, S., Rosado, M.M., Weber, H., et al., Human immunoglobulin M memory B cells controlling *Streptococcus pneumoniae* infections are generated in the spleen, *J. Exp. Med.*, 2003, vol. 197, pp. 939–945. https://doi.org/10.1084/jem.20022020
7. Ezdakova, I.Yu. and Kapustina, O.V., Identification of bovine blood B cells with the immunoperoxidase staining method, *Russ. Agric. Sci.*, 2018, vol. 44, pp. 361–364.
8. Sidorova, E.V., What we know about B-cells today, *Usp. Sovrem. Biol.*, 2006, no. 3, pp. 227–241.
9. Brynjolfsson, S.F., Mohaddes, M., Karrholm, J., et al., Long-lived plasma cells in human bone marrow can be either $CD19^+$ or $CD19^-$, *Blood Adv.*, 2017, vol. 1, no. 13, pp. 835–838. https://doi.org/10.1182/bloodadvances.2017004481


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