ISSN 1068-3674, Russian Agricultural Sciences, 2018, Vol. 44, No. 4, pp. 361–364. © Allerton Press, Inc., 2018.
Original Russian Text © I.Yu. Ezdakova, O.V. Kapustina, 2018, published in Rossiiskaya Sel'skokhozyaistvennaya Nauka, 2018, No. 3, pp. 40–43.

VETERINARY

Identification of Bovine Blood B Cells with the Immunoperoxidase Staining Method

I. Yu. Ezdakova[a], * and O. V. Kapustina[a]

[a]*Skryabin and Kovalenko All-Russia Research Institute of Experimental Veterinary, Federal Science Center, Russian Academy of Sciences, Moscow, 109428 Russia*

**e-mail: ezdakova.i@viev.ru*

Received February 21, 2018

Abstract—The mechanism of formation and the receptor-binding and functional properties of the B-lymphocyte subpopulations in the various immune pathologies are the factors that can affect the basic principles of initiation of a specific immune response and its relationship with the innate immunity of an organism. Identification of the positions of the immunoglobulin receptors on the lymphocytes is the specific clinical information on more accurate disease pathogenesis and differential diagnosis. The survey has proven that the IgM C_2 and C_9 monoclonal antibodies interact with the bovine blood B-lymphocytes. The relative numbers of B cells in the animal peripheral blood and the variation in the positions of the Ig receptors have been determined with the method for direct immunoperoxidase staining.

Keywords: B cells, monoclonal antibodies, membrane-bound forms of immunoglobulins, immunoperoxidase staining

DOI: 10.3103/S1068367418040079

INTRODUCTION

The term *B cells* is formed by the first letter in the English name for the organ where these cells are developed; the B refers to the bursa of Fabricius, an organ in birds, and the bone marrow in mammalia. The B cells differentiate into the antibody producing cells and the memory cells. In addition, they can regulate the various immune responses through producing the cytokines and the other mediators of the immune system. The mechanism of formation and the characteristics of the receptors and functions of B-lymphocyte subpopulations in the various immune pathologies are the factors that can affect the basic principles of initiation of the specific immune response and its relationship with the innate immunity of an organism.

B cell differentiation is related with the direct modulation and reduction of the receptors providing a mechanism for their selection. Density of the cell receptors locating on the B-lymphocyte surface and entering the complex of the transmembrane molecules of immunoglobulin M (IgM) plays a key role in providing the pre-b-cell subpopulations. Three components: the membrane-bound form of IgM (sIgM) and two polypeptides Igα and Igβ (CD 79) are involved in the formation of the antigen B-cell receptor (BCR). Occurrence of these polypeptides on the cell membrane starts at the pre-B-cell stage [1].

Study of the immune responses makes it clear that their components, for instance, Ig-like receptors in this very case, can have dual, often interchangeable function. Thus, B cell sIgM and Igβ may transmit the antigen signals independently from each other. The BCR function is expressed as antigen bounding and transmitting the signal into the cell for the further lymphocyte differentiation and proliferation. The IgM heavy μ-chain is found in the pre-B-cell cytoplasm. A naive B cell expresses the fully displayed molecules of this immunoglobulin on its surface. In addition, the circulating follicular and extrafolicular B cells can transport sIgM on their membranes. The cytoplasmic portion of IgM monomer is composed of three amino-acid residues, which are not sufficient for forming the structural motifs. Therefore, the signal is transmitted via the associated Igα and Igβ glycoproteids, which undergo phosphorylation. Membrane IgMs are oriented towards the external region by the Fab fragment, while the Fc fragment interacts with the cell surface. The SIgM heavy chains contain the domains made of the hydrophobic amino acids at the C-terminal ends, which hold the Ig molecule on the membrane surface. In ontogenesis, the differentiation into the mature B cells is followed by changing the phenotype of the lymphocyte surface, which can be determined with the monoclonal antibodies by the immunocytochemical methods (immunoperoxidase staining). The IgM cytoplasmic expression on the cell surface plays a key role in the B-cell development. The sIg amount modulating or blocking with the antibodies can lead to a change in the immune response pattern in the organ-

ism, since B-lymphocytes concentrate the antigen with their help for subsequent processing in endosomes.

The previously performed experiments have proven the presence of the B cells displaying the globulins at the various surface areas in the bovine and sheep blood [2]. Most currently known immunocompetent cell surface proteins, including sIgM, are the receptors of the contact interaction, which can transform the entering signal into certain cell responses and play a key role in the antigen presentation and the antigen-specific activation of lymphacytes. Identification of the positions of the immunoglobulin receptors on the lymphocytes serves as valuable clinical information on more accurate disease pathogenesis and differential diagnosis. It should be noted that many mechanisms of the lymphacyte sIg expression and the effects of the external and internal factors on the formation of the receptor profile have not been well studied and are still unknown.

The objective of the survey was to determine the B-cell numbers in the bovine peripheral blood with the IgM monoclonal antibodies (mAbs) by the method for immunoperoxidase staining (IPS) [3].

MATERIALS AND METHODS

More than 100 blood samples obtained from the Variegated-and-White cattle aged from 6 months to 5 years were used in the experiment. The blood lymphocytes were isolated with the method of Histopaque 1.077 density gradient centrifugation at 3000 rpm for 45 min. The mononuclear cell concentration in the suspension reached up to $1.0–0.5 \times 10^6$ cells per mL. The blood B cell numbers were determined with the direct IPS method. In order to remove the exogenous immuniglobulins, the 100-μL suspension of the blood mononuclear cells was treated with a 1% solution of lemon acid for 1 min, which was followed by centrifugation in a fivefold volume of a phosphate buffer (pH 7.2) at 1100 rpm for 5 min. The cell suspension on a slide was fixed with ethanol. Peroxidase was blocked with 1% hydrogen peroxide for 10 min. The 10% solution of the horse serum was used as the blocking solution. It was incubated in the humidity chamber at room temperature for 60 min. The 3-Amino-9-ethylcarbosole staining kit (Sigma AEC kit) was used for visualization of peroxidase. The AEC positive cells were identified by reddish brown stain in visualizing the preparations under a microscope (×1000). Peroxidase conjugates of monoclonal antibodies produced in clones C_2 and G_9 were prepared with the method of P.K. Nakane et al. (1974).

RESULTS AND DISCUSSION

The analysis of the mAb immunochemical properties has proven that the monoclonal antibodies have a high efficiency for identifying the IPS B cells that can recognize the conformational antigenic determinants. As the immunoblotting results prove, mAb C_2 and G_9 are only bound with the IgM native molecule, which can indicate the conformation properties of the antigenic determinants in the IgM molecule. Therefore, they were conjugated with the horseradish perozidase in order to use them in the direct IPS variant.

The microscopic examination of the preparations has found that the numbers of B cells in the blood of cows aged 4–5 years, which interact with the monoclonal antigens of clones C_2 and G_9, comprise 21.0 (C_2 B cells) and 31.0% (G_9 B cells), respectively. The number of G_9 B cells in the peripheral blood of the calves aged 1 month and the cows aged 6–7 years comprise 16.6–19.2 and 22.8%, respectively. The method for complementary rosetting was used for comparison, which indicated a 12.0% zymosan-complex rosette-forming B-cell (ZC RFC) fraction in the calf blood. Therefore, the monoclonal antibodies to the transmembrane IgM recognize the B-lymphocytes 37.5% more efficiently than the antibodies to the complement component C3.

The study of variations in the positions of sIg on the B cells is, therefore, of both theoretical and practical interest. The mechanisms of differentiation of the B-lymphocytes into the plasma cells and their antigen-binding capability may be determined by the redistribution of the receptors on the membrane and inside the cell to characterize the functional immunocyte activity [4]. The specificity and the density of the Ig-like receptors play a key role in the B-cell selection and differentiation. The lymphocytes' activation occurs after cross binding of slg.

Receptor-ligand binding is followed by changing the configuration of the receptor and its movement on the cell membrane. Thereafter, the receptors may collect into the *patch* and *cap* structures on the cell surface for subsequent sinking into the cytoplasm and undergoing endocytosis. Surface immunoglobulins similar to the other surface proteins can pass freely through the lipid bilayer of the cell plasma membrane. After the antigen complex formation, they concentrate on one of the poles of the cell to form the clusters that are involved in their cytoplasmic compartment via the cell endocytosis to be degraded (the capping effect). In addition to the mechanism of receptor-ligand binding, there is a phenomenon of shedding the receptors. The clusters composed of the aggregated antigen-sIg complexes on one of the cell poles are separated from the cell surface and released into the extracellular medium. Both endocytosis and shedding are the essential parts of the common function of the cell receptor apparatus.

Studying the variation in positions of the membrane-bound immunoglobulins on the B cells in the young and adult animals is a future direction for the prospective research into the cellular mechanisms of

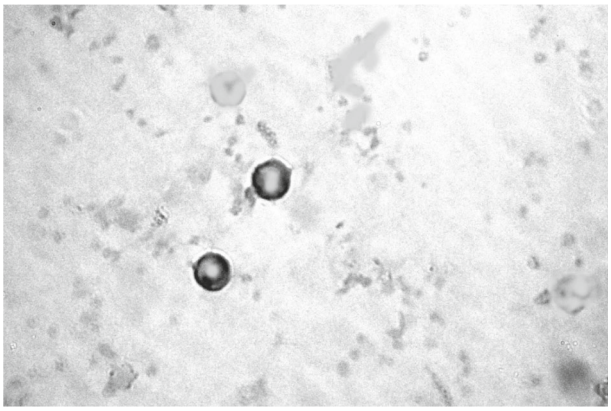

Fig. 1. Calf C_2B cells (of *ring* type), ×1000.

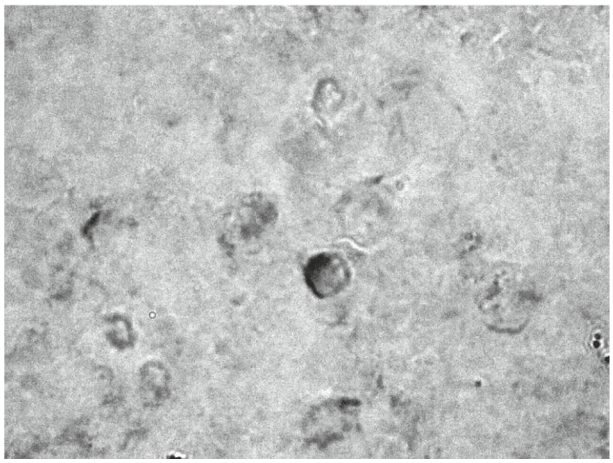

Fig. 2. Calf B cells (of *patch*/endocytosis type), ×1000.

activation and energy intake during ontogenesis and immunogenesis.

The previous surveys have proven that lymphocytes with the membrane stained during the formation of the *patch*/endocytosis, *patch*, *ring*, and *cap* types to detect the population of B cells in the cows aged 4–5 years comprise 68.9, 15.0, 10.5, and 5.6% [5]. The current survey has determined that C_2B cells comprise 9.3% in the blood of the calves aged 6 months, which are present in the forms of the *patch*/endocytosis (83%) and *ring* (65%) types (Fig. 1), while the number of the G_9B-lymphocytes comprises 20.4%, including 93% *patch*/endocytosis (Fig. 2) and 1% *ring* form types.

Differentiation of B-lymphocytes into the plasma cells and the B memory cells is followed by intensive mutagenesis. Morphologically, there is no difference between the memory cells and the naive small lymphocytes, while they are different in the phenotype and the receptor specificity. The memory B cells carry receptor immunoglobulins of classes IgG or IgA but not IgM or IgD typical for the naive B-lymphocytes. The receptors of the memory B cells are determined more relative to the antigen, since these cells have passed through the selection processes within the germinal centers. However, the molecular mechanisms of more efficient response of the memory cells are not completely ascertained yet.

Three subpopulations of the bovine B-lymphocytes: B1a $CD5^+$, B1b $CD5^-$, and B-2, possessing the various surface receptors and the functional properties but expressing the membrane form of immunoglobulin M are known at present. The antibodies produced by the B1 lymphocytes are generally referred to IgM. These lymphocytes are destined to the rapid response to the most distributed antigens of the bacterial cell walls. The greatest population is presented by the B-2 lymphocytes, which can differentiate into the plasma cells and produce the immunoglobulins of all the well-known isotypes. The antigen type can affect the switch in the isotypes. Thus, the bacterial cell surface carbohydrates can induce the IgG3 production in mice, while the viral infection generally induces the production of the IgG2a-isotype antibodies [6]. In addition, this process may occur without any impact of the exogenous antigen during the early colonial expansion and the development of the B cells. The surface immunoglobulins of different isotypes on the same B cell possess the same antigen specificity. The B-lymphocytes carrying the immunoglobulins of different isotypes have been defined to possess distinct functions in the animal organisms. Thus, sIgA B cells participate in the lymphocyte migration to the mammary gland in cows, while the IgG plasma cells play a key role in the immune response to the helminth infection.

Under the antigen effects, the B-lymphocytes start the intensive process of fission, the immunoglobulins of Ig classes—IgG and less common IgA and IgE instead of IgM and IgD typical for the naive B cells—occur on the cell surface, and the mutation frequencies are increased [7]. This can cause significant new variations in the receptors of the B-lymphocyte subclones. They can die if the mutations cause the low affinity of the receptor for the antigen and the B cells do not receive the inflammatory signals from the dendritic cells acquiring the antigen. In case of a high affinity of a receptor to the antigen, the cells survive, leave the follicle, and migrate into the lymph nodes, the spleen, and the bone marrow (especially at the secondary response), where they differentiate into the plasma cells and secrete antibodies. The B cell stimulation in the Peyer's patches can result in migration to the submucous layer of the gastric mucosa and the subsequent differentiation into the IgA secreting plasma cells. The antibodies to the different antigenic determinants are the soluble forms of the B-cell membrane immunoglobulins, namely homologues of the antigen-binding sites of the B-cell sIgM receptor.

The analysis of the experimental data sets has allowed us to draw a conclusion that mAb G_9 and C_2

differ in the density and the strength of binding with the B-cell Ig receptor. The antigen determinants interacting with the antibodies of clone C_2 are probably located in the region of the nonhomologous sites of V set domains in the immunoglobulin molecule and are not always accessible for binding. This is explained by the frequently observed substitutions and deletions of the amino acid residues in the Ig molecules that are associated with their spatial structures. mAb G_9 are probably focused on the epitope in the homologous region of the polypeptide chain; thus, they are a better fit for IPS.

CONCLUSIONS

Therefore, the survey results have proven that the relative numbers of the B cells in the blood of the cows aged 4–5 years and 6–7 years comprise 31.0 and 22.8%, respectively, while they comprise 16.6–19.2 and 20.4%, respectively, in the calves aged 1 month and 6 months. The data sets obtained during the survey will allow us to determine the statistical parameters for the bovine blood B-lymphocytes during ontogenesis and immunogenesis. At present, there is no doubt about the fact that the quantitative characteristics of the animal blood B cells are considered indispensable, since these lymphicytes are the most important immunoregulatory cells that can affect the immune system's status.

REFERENCES

1. Nutt, S. L., Hodgkin, P.D., Tarlinton, D.M., and Corcoran, L.M., The generation of antibody-secreting plasma cells, *Nat. Rev. Immunol.,* 2015, no. 15, pp. 160–171.
2. Ezdakova, I.Yu., *Retseptory immunnogo uznavaniya u zhivotnykh* (Receptors of Immune Recognition in Animals), Moscow: Kompaniya Sputnik+, 2008.
3. Sidorova, E.V., What do we know about B-cells today?, *Usp. Sovrem. Biol.,* 2006, vol. 126, no. 3, pp. 227–241.
4. Ezdakova, I.Yu. and Popova, E.V., Immunoglobulin receptors of animal B-lymphocytes, *Tr. Vseross. Naucho-Issled. Inst. Eksp. Vet. im. Ya. R. Kovalenko,* 2015, vol. 78, pp. 199–206.
5. Ezdakova, I.Yu., Localization of IgM in B cells of peripheral blood of cattle, *Allergol. Immunol.,* 2008, vol. 9, no. 3, p. 274.
6. de Jong, B.G., Ijspeert, H., and Marques, L., Human IgG2- and IgG4-expressing memory B cells display enhanced molecular and phenotypic signs of maturity and accumulate with age, *Immunol. Cell Biol.,* 2017, pp. 744–752.
7. Tolar, P., Cytoskeletal control of B cell responses to antigens, *Nat. Rev. Immunol.,* 2017, vol. 17, pp. 621–634.

Translated by O. Zhiryakova