The use of poly- and monoclonal antibodies to assess the state of the immune system

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Abstract. The function of the immune system of animals is influenced by a fairly large number of external (environmental, anthropogenic, infectious) and internal (mutations, impaired protein synthesis) factors. The result of their exposure is either the activation of the entire system or its individual links, or its suppression, causing the development of an immunodeficiency state. The quantitative determination of proteins of the immunoglobulin superfamily, namely, natural antibodies and cell receptors, provides valuable clinical information for clarifying the pathogenesis of the disease and differential diagnosis. The immunoglobulin profile of the body determines the functional ability of the immune system to recognize foreign agents. B-cell membrane immunoglobulins are the receptor for the antigen and are identical to its secreted form - the antibody. The modulation of their number reflects the processes of activation and inhibition of cellular reactions. The cell is constantly recycling membrane proteins. Similar processes occur with immunoglobulins, some of which are immersed in the cell, while others, on the contrary, are built into the membrane or secreted into the extracellular space in soluble form. Immunodiagnostics based on the determination of the immunoglobulins that make up the membrane and soluble pools makes it possible to assess the functional state of not only B cells, but also the immunoreactivity of the body as a whole. This paper presents a quantitative assessment of the soluble and membrane forms of clinically healthy cattle immunoglobulins using poly- and monoclonal antibodies.

1. Introduction

To evaluate any component of the immune system, a large set of tests is proposed to identify various stages of development and the components of this system. It is important to choose a set of tests that allows you to get the most important information about the state of the immune system. Immunodiagnostics requires the use of an individual set of tests to assess the functional state of individual parts of the immune system. Using these methods, it would be possible to solve problems of a very wide spectrum - from making a laboratory diagnosis of immunodeficiency to elucidating the functional state of T-, B- and phagocytic immunity [1].

Most proteins with the participation of which antigen recognition occurs belong to the immunoglobulin superfamily (IgSF). They contain common structural elements and are characterized by a specific spatial domain organization and statistically significant homology. Ig domains have a sandwich structure, which is constructed from antiparallel β-folded sheets joined by disulfide bonds. Disulfide bonds are located in characteristic places of the peptide chain and can be intra- and
intermolecular. The common functions of the proteins of this family are the ability to specifically recognize and distinguish macromolecules during cell interaction carried out using leukocyte membrane receptors [2].

Representatives of IgSF are immunoglobulins (Ig), proteins of the main histocompatibility complex (MHC), T and B cell receptors, Fc receptors, membrane antigens (CD), cell adhesion molecules, etc. With the exception of secreted forms, almost all superfamily proteins have transmembrane segments at the C-end of the molecule that can be inserted into the membrane, they are called immunoglobulin-like leukocyte receptors - LIR (leukocyte Ig-like receptor).

Antibody molecules have the most complex structure in this superfamily. The following are distinguished in the structure of immunoglobulin: constant regions (constant sequence of amino acids); variable parts; hypervariable sites; the hinge regions necessary to change the conformation, in which there are sites of binding of proteins of the complement system. When processing an immunoglobulin molecule with papain, it breaks down into three fragments - 2 Fab fragments (Fragment antigen binding) and 1 Fc fragment (Fragment crystallisable) fragment. The C-terminal portion of the H-chains serves to attach to the cellular Fc receptor [3,4].

Both Fab fragments consist respectively of a single L chain and an N-terminal portion of the H chain. Isolated Fab fragments retain the ability to bind antigen. The Fc fragment consists of the C-terminal half of both H-chains. This part of Ig performs the functions of binding to the cell surface, interacting with the complement system and is involved in the transfer of antibodies by cells. The Fc fragment is able to activate the complement system, provides the ability of immunoglobulin G to pass through the biomemran (passes through the fetoplacental barrier in some mammals), determines cytophilicity, and has an opsonizing effect on phagocytosis objects. Any immunoglobulin has at least 2 active centers, and if molecules are combined into a polymer - and more.

Despite the wide variety of forms, the general principle of structure is observed in immunoglobulins. IgG heavy peptide chains (H-chains) consist of four globular domains V_H, C_H1, C_H2 and C_H3, and light (L-chains) consist of two globular domains CL and VL. Both heavy chains, as well as the light and heavy chains, are linked by disulfide bridges that stabilize the tertiary structure within the domains. Domains have a length of about 110 amino acids and have mutual homology. In the central region of the immunoglobulin molecules, there is a hinge region that provides antibodies with intramolecular mobility.

By the nature of the Ig-domains, surface molecules can be divided into three groups: receptors formed by V-domains (CD8, CD28, etc.); receptors formed only by C2-domains (ICAM 1,2,3 and others); receptors containing V and C2-domains (MHC II, CD2, CD4 antigens, etc.). C-terminal regions of the (cytoplasmic) receptors are associated with phospholipids of the cytoplasmic membrane, are associated with protein kinases and contain phosphorylation sites for these enzymes.

IgSF glycoproteins are involved in the humoral and cellular immune response. At the same time, the humoral mechanisms of the immune system provide soluble forms of immunoglobulins, and cellular - membrane-bound Ig receptors of immunocompetent cells.

Cell surface receptors belonging to the family of immunoglobulins are directly involved in the recycling of mature lymphocytes, in the presentation of antigen, antigen-specific activation of lymphocytes, in chemotaxis and phagocytosis, in the implementation of all types of cell cytotoxicity, in the regulation of non-lymphoid cell functions by lymphocytes. Therefore, their identification and characterization reflect the state of the immune system at a given time.

The aim of the study was to determine the soluble and membrane forms of Ig immunocompetent cells using poly - and monoclonal antibodies.

2. Materials and methods

In the experiments, samples of biological fluids (blood serum, nasal secretion, blood) of cattle were used. To obtain immunochemically pure immunoglobulins in cattle, chromatographic sorbents Sepharose 6B, Sephacryl S-400, S-300, DEAE-Sepharose 6B were used; immunoglobulin isotyping kit ("Sigma"). To obtain a polyclonal antiserum, rabbits of the breed "Soviet Chinchilla" with a mass
of 2.5-3.0 kg were immunized. 0.5 ml of Freund's complete adjuvant mixed with an equal volume of Ig was injected into the popliteal lymph nodes. After 7 days, 0.5 ml was re-injected into each popliteal lymph node. The double radial immunodiffusion reaction was used to determine purity, working dilution of antisera and isotyping of antibodies. To control the specificity of antisera, the method of immunoelectrophoresis was used. Monoclonal antibodies to IgM and IgA in cattle were obtained by the method of G. Köhler and C. Milstein.

The concentration of immunoglobulins of classes G, M, and A was determined in a simple radial immunodiffusion (RID) reaction and enzyme-linked immunosorbent assay (ELISA) using polyclonal sera to IgG and IgA we obtained and monoclonal antibodies (Mabs) to cattle IgM and IgA.

Cell isolation was performed using nutrient media 199, RPMI-1640 and fetal cattle serum. Cells were separated in a density gradient of Histopaque-1077 (Sigma). Cell viability was determined using trypan blue. For immunoperoxidase staining (IPO) of the cells, solutions of citric acid, hydrogen peroxide, BSA, horse serum, rabbit, 3-amino-9-ethylcarbazole (SEC “Sigma”), poly-L-lysine were used. Membrane forms of IgSF proteins of immunocompetent cells were identified using monoclonal antibodies to CD19 and IgM.

Immunoperoxidase staining of cells. Peroxidase blockade on glasses with fixed cells was carried out with a 10% hydrogen peroxide solution for 10 min. As a blocking solution, a 10% -15% solution of horse or rabbit serum on PBS was used. Incubated for 60 minutes, at room temperature and cells were washed thoroughly. Then monoclonal antibodies were added to the fixed cells [5]. To visualize peroxidase, a staining kit with 3-amino-9-ethylcarbazole AEC (Sigma) was used, washed in PBS and examined under a microscope (x400, 1000).

3. Results and discussion

The number of soluble forms of immunoglobulins was determined by the method of simple radial immunodiffusion according to the Mancini method and in an enzyme-linked immunosorbent assay using the polyclonal sera to IgG and IgA we obtained and monoclonal antibodies (Mabs) to cattle IgM and IgA.

The use of monoclonal antibodies in RID suggests the presence of precipitating properties of Ig. As a result of the studies, clones C2 and G9 were produced that produce antibodies that interact with the native IgM molecule and retain the titer in the double radial immunodiffusion reaction (1:32) after lyophilization.

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To assess the level of IgA, along with RID, an enzyme-linked immunosorbent assay (ELISA) based on monoclonal antibodies directed to two non-competing epitopes on the Ig molecule was also used, which ensures high sensitivity and specificity of the test system. [6]. The analysis showed a partial correlation of the results. In addition to negative, a positive correlation was observed in blood serum, which can be explained by the presence of small amounts of dimeric and polymeric forms of serum IgA. In tear secrets, the absence of correlation is apparently due to the presence of a greater variety of polymer forms, compared with similar forms in nasal secretions.

Polyclonal antisera have antigenic specificity, whereas Mabs is epitopic. When immunizing laboratory animals with immunochemically pure Ig, a wide range of antibodies to the antigenic determinants of the immunoglobulin molecule were obtained in antiserum. As a result of the studies, it was shown that the use of polyclonal antibodies is preferable in RID for the quantitative determination of class G immunoglobulins. The concentration of class M and A immunoglobulins is more reliable in ELISA using monoclonal antibodies directed to several conformational epitopes on the Ig molecule.

As a result of the studies, it was found that the concentration of IgG in the blood serum of cows is $23.06 \pm 2.05$ mg/ml, IgM - $2.6 \pm 0.3$ mg/ml, IgA - $1.06 \pm 0.2$ mg/ml In nasal secretions, the level of sIgA - is $2.1 \pm 0.1$ mg/ml.

The data obtained can be of practical importance in organizing activities aimed at improving the prevention of infectious diseases. The level of immunoglobulins in biological fluids of the body has
an important prognostic value during vaccination, so in animals with low rates an inadequate immune response is possible, which manifests itself in an insufficient level of formation of specific protective antibodies [7].

Vital activity of cells is accompanied by a drift of membrane proteins. CD-receptors are markers of the functional state of cells in a certain period of time. The quantitative characterization of these molecules can serve as a diagnostic guideline not only for assessing the immune status, but also for studying the mechanisms of the immune response in various animal diseases. Using poly- and monoclonal antibodies to proteins of the immunoglobulin superfamily, it became possible to study the localization of sIg and determine the number of T- and B-lymphocytes.

The functions of IgSF membrane proteins mainly consist in the implementation of intercellular interactions and the conduct of an antigenic signal. Four types of Fc receptors for immunoglobulins are expressed on the membrane of immunocompetent cells, through which Ig-mediated reactions are carried out.

The surface is a kind of "passport" of the cell, by which you can identify the type and function of the lymphocyte. The main membrane molecules involved in the recognition of antigens belong to the superfamily of immunoglobulins. Membrane-bound Ig and antibodies are signaling molecules by which the main adaptive immune responses of the body are carried out. We believe that the proteins of the immunoglobulin family both on the cell surface and in the circulating blood form a kind of immune control system for the surfaces of not only immunocytes, but also cells of the nervous and endocrine systems.

It should be noted that many mechanisms of sIg expression of lymphocytes, the influence of external and internal factors on the formation of the receptor profile of the cell are poorly understood and are still unknown.

In this regard, the next stage of work was the optimization of the immunoperoxidase staining reaction using mono- and polyclonal antibodies to cattle Ig. Immunoglobulins in the body can be in membrane-bound (sIg), cytoplasmic (cIg) and secreted (Ig) forms, which are also an indicator of the level of differentiation of B-lymphocytes.

IgM monomer is expressed on the surface of the B-lymphocyte, which is an integral part of the B-cell receptor complex for antigen. Under the influence of the local microenvironment, the precursors of B-lymphocytes (large pre-B cells) divide, forming pre-B cells containing heavy chains of class M immunoglobulins in the cytoplasm and then acquiring the ability to synthesize light chains. This process is caused by the rearrangement of Ig genes and their expression arising in this phase. It should be noted that there is a lack of synchronism in the restructuring of Ig genes, so the genes of heavy and then light chains are rebuilt at the beginning. This stage is an antigen-independent phase of B-cell differentiation, during which a wide variety of pre-B-cell clones that do not have immunoglobulin receptors capable of interacting with the antigen arise. Then the pre-B cells turn into small immature B cells, on the surface of which there are transmembrane IgM molecules (sIgM), the main function of which is the recognition and binding of various ligands. Three components are involved in the formation of B-cell receptor (BCR): the membrane form of IgM and two Igα, Igβ polypeptides (CD79). The appearance of these polypeptides on the cell membrane begins at the pre-B cell stage. Studying immune reactions, we come to the conclusion that their components, in this case, Ig-like receptors, perform dual functions and are often interchangeable. Thus, surface IgM and Igβ B cells can transmit an antigenic signal independently of each other. The function of the antigen receptor is to bind the antigen and conduct a signal into the cell for further differentiation and proliferation of the lymphocyte. This receptor has significant similarities with the pre-Tα/β receptor of T lymphocyte precursors. Immunoglobulin-like domains connected by disulfide bonds are those structural elementary units from which the body builds an immune recognition system.

In the cytoplasm of pre-B cells, a heavy μ-chain of IgM is detected. A naive B-cell expresses complete monomeric molecules of a given immunoglobulin on its surface. The circulating follicular and extra-follicular B cells also carry sIgM on their membranes [8,9].
The cytoplasmic part of membrane IgM consists of three amino acid residues, which are not enough for the formation of structural motifs; therefore, the signal is transmitted through the associated Igα and Igβ glycoproteins, which undergo phosphorylation. The synthesis of secretory antibodies is provided by differential splicing. The primary nuclear RNA transcript for the μ chain includes sequences encoding hydrophobic transmembrane regions that can be excised by splicing and immunoglobulin molecules are synthesized in soluble form.

Membrane IgMs are oriented by the Fab region toward the external environment, while the Fc fragment is in contact with the cell surface. S-IgM contains at the C-ends of the heavy chains domains formed by hydrophobic amino acids that hold the Ig molecule on the outer surface of the cell.

The expression of cytoplasmic IgM on the cell surface plays a crucial role in B-cell ontogenesis. Modulating the amount of surface Ig (sIg) or blocking them with antibodies leads to a change in the nature of immune responses in the body, since B lymphocytes concentrate the antigen with sIg, which bind it for further processing in endosomes.

sIgM and sIgG B cells were determined by immunoperoxidase staining using monoclonal antibodies to cattle IgM and polyclonal antibodies to IgG as the first antibodies.

4. Conclusion
As a result of the studies, it was found that the peripheral blood cells of calves expressing IgM are 19.2 ± 0.8%. In cows, blood cells with superficial immunoglobulin class M comprise 25.3 ± 2.6%, and with IgG 10.4 ± 0.4%. B cells with sIgG are cells secreting natural antibodies of class G. Cells with sIgM are naive B cells. It was shown that in the blood of calves the number of B-lymphocytes is less than in adult animals, which is associated with the immaturity of the immune system, and correlates with the level of soluble forms of immunoglobulins in blood serum. Also, as a result of immunocytochemical analysis in the blood of calves, cells with a cytoplasmic IgM of 21.5 ± 0.4% were detected. These cells do not stain around the perimeter, i.e. membrane immunoglobulins are absent. Consequently, B cells are present in the peripheral blood of calves at different stages of differentiation.

Thus, using poly- and monoclonal antibodies, the state of the immune system of cattle was assessed based on the determination of proteins of the superfamily of immunoglobulins present in animals as antibodies and cell receptors. The study of the relationship between soluble and membrane forms of Ig in young and adult animals is a promising area of research into the mechanisms of cellular activation and anergy in the processes of ontogenesis and immunogenesis. The study of the activation mechanism of IgSF molecules seems to be extremely important not only for understanding the physiological basis of immunity, but also for controlling these processes under the influence of various factors on the body. The accumulation and analysis of new data on membrane-dependent processes for the recognition of antigens serves as the theoretical basis for the development of immunological test systems for the diagnosis of pathological conditions of various genesis at the molecular level, their prediction and treatment.

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