

SUMMARY

IMMUNGLOBULIN Y- BIOLOGICAL VALENCES AND THERAPEUTIC USES

PhD student: CHIURCIU Constantin

Scientific coordinator: Professor DANEȘ Doina DVM PhD

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The egg yolk is considered to be an ideal source of immunoglobulins, and among them, IgY is the predominant antibody. The literature provides much information on the advantages of using immunoglobulins Y over mammalian IgG.

Firstly, IgY is obtained by non-invasive methods, making it suitable for large-scale manufacturing. Thus, IgY antibodies can easily be gotten by the simple action of egg collection, separation of yolks, isolation and purification instead of the stressful method of animal bleeding in order to obtain the serum. The available amount of a particular antibody is considerably increased by repeatedly obtaining eggs from the same hen. Production of chicken egg antibodies is higher compared to that of a mammal of similar size. Within a year, a large amount of antibodies can be obtained from one single hen, about 40 g, of which 1-10% can be specific antibodies. IgY also offers remarkable economic benefits because the cost of rearing hens is lower than the one for rabbits.

Secondly, due to the phylogenetic distance between hens and mammals, IgY does not interact with rheumatoid factors, with the receptors for the crystallizable fragment (Fc) and does not activate the mammalian complement system.

Also, IgY antibodies can recognize more highly conserved mammalian protein epitopes than the mammalian IgG, so IgY can induce a more effective immune response. If chickens and rabbits are being immunized with the same antigen, birds respond with specific antibodies, which can rarely be achieved in rabbits. Immunization of hens requires small amounts of antigen to obtain a high and sustained IgY titer in the egg. IgY antibodies are stable over time: when stored 10 years at 4 °C no significant loss of activity was recorded; they maintained their activity after 6 months at room temperature or one month at 37 °C.

IgY antibodies play an increasing role in research, diagnosis and therapy. Because of the high amounts of IgY that can be obtained, they can be used in the immunotherapy and/or immunoprophylaxis of many viral and bacterial infections, in both human and veterinary medicine, and as an alternative to classical antibiotherapy. In addition, the immunoglobulins Y can be used as a natural immune supplement in infant formulas or other novel foods.

Based on the advantages of the IgY technology and its applications in various fields, our research program in ROMVAC Company focused on the production of immunoglobulins Y from laboratory to industrial scale, their immunochemical purification and characterization and the demonstration of their specificity against the antigens that were used to immunize the hens. The obtained immunoglobulins Y proved to be specific against the bacterial strains, many of them antibiotic-resistant, isolated from patients with acute and chronic infections from hospitals in Romania, as well as from cows with subclinical mastitides. These immunoglobulins Y have been formulate dunder various dosage forms of the IMUNOINSTANT brand: solution, gel, powder, spray.

Relaying on the results of the *in vitro* experiments - which have shown that specific immunoglobulins Y inhibit the multiplication of antibiotic resistant bacteria - the general objective of the present thesis was to assess the therapeutic spectrum and efficacy of immunoglobulin Y. Thus, the IMUNOINSTANT products, containing monovalent/bivalent/polyvalent IgY, were administered to treat conditions such as subclinical mastitis, psoriasis, periodontitis, methicillin-resistant *Staphylococcus aureus* infections.

THE FIRST PART of the thesis ("*Bibliographic study*") comprising around 50 pages, describes the state of knowledge in regard to: the avian immune system (**Chapter I**); immunoglobulins: structure, functions; chicken immunoglobulins (**Chapter II**); and specific action of fowl immunoglobulins (**Chapter III**).

The primary lymphoid organs of the immune system in birds are the thymus (with a role in the production and maturation of T lymphocytes) and the Fabricius bursa (with an important role in the maturation of B lymphocytes). The secondary lymphoid organs of the immune system in birds are the spleen, the lymph nodes and the uncapsulated lymphoid tissue - located at the digestive, respiratory, urogenital and cutaneous epithelium.

B lymphocytes that provide humoral immunity leave the burs through the blood vessels supplying the bursal follicles, reaching the lymphoid tissues, with an important role in the production of antibodies (immunoglobulins). In birds, immunoglobulins G and M were identified in the blood as main serum antibodies, while IgA was identified in secretions (bile). Literature data suggests an analogy between the passive transfer of immunoglobulins in birds and mammals.

In 1962, immunoglobulin Y was identified by Williams J. as a gamma globulin in y-livetin fraction of egg yolk, and in 1969, due to the major differences between bird and mammalian IgG-s, Leslie GA and Clem LW proposed the term IgY for the IgG in the egg yolk. Despite the functional homology between avian IgY and mammalian IgG, there are differences in molecular weight, structure, and biochemical functions. The true egg yolk immunoglobulin, IgY (γ -livetin) has become the subject of many investigations to assess its potential as a prophylactic and therapeutic agent in infectious pathologies, especially for the treatment of antibiotic-resistant pathogens. It can be used as an alternative to classical antibiotherapy, both in human and veterinary medicine. In the veterinary field, administration of immunoglobulin Y has been shown to be successful in the treatment of a variety of gastrointestinal infections with bovine rotavirus, bovine coronavirus, *Yersinia ruckeri*, enterotoxigenic *Escherichia coli*, *Salmonella spp.*, *Edwardsiella tarda*, *Staphylococcus* and *Pseudomonas*. In terms of prophylactic use of IgY, this is an alternative to mammalian antibodies for oral passive immunization against various enteric pathogens. In human medicine, therapeutic IgY administration may reduce the clinical use of antibiotics and minimize the risk of bacteria developing resistance to antibiotics. Immunotherapy can be used against pathogens that are difficult to treat with traditional antibiotics. IgY has been used in the treatment of: intestinal infections in children, colitis and celiac disease, cystic fibrosis, gastric ulcer and tooth decay. It has also been used to prevent the avian/swine influenza pandemic, obesity, to control staphylococcal enterotoxin B (SEB) - a potential biological weapon - and in proteomics. IgY antibodies are also used in other applications including: diagnosis of gastric cancer, detection of ovarian and breast cancer markers, detection of AHS virus, determination of hepatocyte growth factor (HGF) in serum and urine, diagnosis of infections with *Campylobacter fetus* and *Bordetella bronchiseptica*, detection of TGF (Tumor Growth Factor) in biological fluids, determination of inflammation reagents, and detection of human serum antigens using surface plasmon resonance (SPR). Antibodies IgY anti-E7 HPV16 have also been obtained, making it possible to use them in the screening of clinical samples, as well as anti-venom IgY antibodies..

PART TWO, “*Personal research*” begins with the general purpose and objectives of the research, continues with the presentation of the processes for obtaining and formulation of immunoglobulin Y from hyperimmune eggs, then with the presentation of several case studies describing the therapeutic applications of these products in humans and animals, and ends with the general conclusions and recommendations, as well as with the references. Along its four chapters (IV to VII), representing approximately 67 % of the volume of the thesis, this part contains 32 tables and 79 pictures.

Chapter IV describes the *production, control and formulation of egg immunoglobulins Y* performed in the IMUNOINSTANT research and manufacturing laboratory, owned by ROMVAC Company.

The procedure for obtaining the specific immunoglobulins Y includes: 1) the methods of isolation and identification of bacterial and/or fungal strains collected from patients who have been treated with antibiotics but without success; 2) antigen processing for inoculation of hens (monovalent and polyvalent); and 3) the immunization scheme of conventional or SPF hens from which the hyperimmune eggs were collected.

In the subchapter on control of specific immunoglobulins Y, the following tests used for assessing their activity/specificity are presented: 1) indirect ELISA, 2) direct ELISA, 3) agar gel immune - diffusion test (AGID), 4) Radial Immunodiffusion Test (RIT), and 5) Rapid Slide Agglutination Test (RSAT).

The next subchapter describes the manufacturing steps and technology for obtaining the immunoglobulin Y dosage forms (solution, powder, spray, suspension, ointment and gel), and a brief presentation of the composition and intended use of several IgY containing finished products.

The last subchapter presents the use of IgY obtained from hyperimmune eggs for the production of IgY-based reagents designed for *in vitro* assays such as ELISA or immunofluorescence.

Chapter V, "*The use of specific egg immunoglobulins in the treatment of human morbid entities, which have exclusive palliative therapy*" consists of two subchapters (5.1. Psoriasis and 5.2. Periodontitis), each comprising: 1) a general presentation of the condition (definition, causes, treatment, prognosis), 2) the applied treatment, 3) the results/discussions, and 4) conclusions. The studies were conducted in patients with psoriasis and periodontitis, in the IMUNOINSTANT Complementary Medicine Cabinet and in a dental office.

Psoriasis is a disease of an unclear etiology, which reduces the quality of life and causes many psychological and psychosocial problems in the affected individual. However, it has been established that genetic predisposition and environmental stimuli contribute to immunological, biochemical and vascular abnormalities, leading to proliferation and aberrant epidermal differentiation. In order to improve the quality of patients' life, the applied therapeutic regimens were perfected, but without healing the disease.

Antibodies are a new hope for the management of psoriasis, offering advantages such as: lack of toxicity to internal organs, excellent tolerability, and induction of sustained remission. The IMUNOINSTANT products containing egg yolk polyclonal IgY antibodies may be a chance for patients with psoriatic lesions infected with specific, susceptible or antibiotic-resistant pathogens. IgY products did not induce any adverse effects or idiopathic immune responses in patients, regardless of their age, the only exclusion criterion being the allergy to egg proteins. The patients included in our studies were clinically diagnosed with psoriasis vulgaris, with different localizations and at different stages. The treatment was initiated based on the physical and clinical evaluation. The assessment of the therapeutic effects was performed by: checking the

improvement/remission of psoriasis symptomology, histological examination, and microbiological examination of the presence/absence of originally identified pathogens. In all presented cases, general and personalized, oral and topical therapy with products containing polyvalent/bivalent or monovalent Y immunoglobulin was found to be effective over a long term, as demonstrated by the good, promising results in most of our subjects with psoriasis. From the structural point of view, both the skin and the nails of the patients have returned to normal.

Periodontitis or marginal parodontopathy is a dental condition that defines the illness of the marginal periodontium initiated and perpetuated by various bacteria (*Streptococcus mutans*, *Streptococcus mittis*, *Streptococcus sanguinis*, *Streptococcus salivarius*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Tannerella forsythensis*, *Fusobacterium nucleatum*, *Bacteroides forsythus*, *Actinobacillus actinomycetemcomitans*). It occurs due to alteration of the balance between the defense capability and the local/general resistance of the organism, on one hand, and the harmful action of extrinsic and intrinsic factors of the periodontium, on the other hand. A more secure approach to dental disease control than the active immunization is the local passive immunization based on murine monoclonal antibodies, plant transgenic antibodies, IgY antibodies from egg yolk and bovine milk antibodies against specific bacterial antigens. Oral immunotherapy with IgY is a promising new treatment strategy for infections in the oral cavity. A 0.1% IgY solution can reduce the weight of the plaque on the tooth surfaces by 21.1% as compared to the initial moment, while oral administration of IgY anti-*Candida albicans* significantly reduced the number of yeasts, the lesions on the tongue, the colonization of yeast in other organs, thus reducing its dissemination due to blocking the binding of the microorganism to the host cells.

In all our case studies, the oral and topical application of personalized IMUNOINSTANT IgY antibodies, led to promising results. Post-therapy microbiological analyzes showed negative results for the pathogens isolated prior to treatment: *Fusobacterium nucleatum*, *Enterococcus* spp., *Enterobacter* spp, *Streptococcus* spp. α -hemolytic, *Streptococcus* group B, *Streptococcus* group D and *Staphylococcus* spp. α -hemolytic. The results obtained with our IMUNOINSTANT and IMUNODENT products, support the literature data concerning the efficacy of monovalent/bivalent/polyvalent IgY treatments in reducing the bacterial colonization of the oral cavity.

Chapter VI," *The use of specific egg immunoglobulins in the treatment of human/animals morbid entities, which have exclusive curative therapy* [6.1. *Staphylococcus aureus* methicillin - resistant (MRSA) and 6.2. Subclinical mastitis], structured as: 1) general presentation (definition, causes, treatment, prognosis), 2) applied treatment, 3) results/discussions and 4) conclusions.

MRSA infections are commonly contracted in the hospital or in the community and frequently cause diseases in people with a compromised immune system. MRSA is a bacterium resistant to a lot of antibiotics, making it difficult to treat it. In our studies

on skin infections present in psoriasis wounds, 36.6% were found to be *Staphylococcus aureus* and 21.9% MRSA. The large-scale use of antibiotics in the feed industry for preventive/ curative treatment of diseases and growth promotion has led to both the development of antibiotic resistant bacteria and the antibiotic inefficiency. Specific antibody immunotherapy is a good alternative to antibiotics, and specific immunoglobulins Y are good candidates to replace antibiotics in fodder or drinking water. Experiments have shown an inhibition of the growth of *Staphylococcus aureus* by specific IgY at concentrations of 1-5 µg/ml, probably due to the interaction with the surface components of the bacterium.

Our studies, performed in the IMUNOINSTANT Complementary Medical Office, focused on patients with urinary infections, localized fistulae, postsurgical/diabetic wounds, dermatological disorders (acne, epidermolysis) mostly caused by MRSA. The treated patients had different ages (7-72 years), with infections of different stages, ages and locations. In the presented cases, oral and topical administration of IgY-containing products (PC2 hyperimmune eggs, IMUNOINSTANT (solution, spray, gel), IMUNOINSTANT G, UNGUENT PV) to MRSA-infected patients resulted in favorable outcomes after several months of general and personalized treatments (1-10 months).

Mastitis is an inflammatory process of the mammary gland, which causes physico-chemical and pathological changes of both the glandular tissue and milk. It is a serious problem for the dairy industry, with losses of up to 10-15% of the annual cow milk production; in Romania, the losses due to mastitides are estimated at about 11%. In subclinical mastitides, the milk does not undergo obvious changes, it appears to be normal, but somatic cell and “acceptable” bacteria counts exceed the maximum allowed, pathogens are recorded, the production of milk decreases and it coagulates upon boiling. Besides production, mastitides affect animal welfare and human health. Among the pathogens involved in cow mastitis are *Streptococcus uberis*, *Streptococcus agalactia*, *Staphylococcus aureus*, *Streptococcus dysgalactia*, *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Serratia liquefaciens*, *Serratia rubidaea*, *Pseudomonas aeruginosa*, *Mycoplasma agalactiae*, *Nocardia nova*, *Nocardia farcinica* a.s.o.

The treatment of this condition is a major problem due to the costs and the antibiotic resistance caused by the wrongly applied treatments. Currently, the treatment of mastitides is based on antibiotics, but these often become ineffective against pathogens; immunotherapy/immunoprophylaxis are becoming the solutions of choice. There are commercially available vaccines against several mastitis causative agents, but they are mainly used for immunoprophylaxis, being less effective in therapy. An attractive alternative is the IgY antibody therapy which proved to reduce the somatic cell count, inhibited bacterial growth and increased phagocytosis of pathogens by milk macrophages. We have developed a product (IMUNOINSTANT-VOC) containing hyperimmune egg yolk derived IgY against microorganisms isolated from cows on a farm experiencing this condition for several years (caused by pathogens no

longer responding to antibiotics). Our tests aimed at assessing the preventive and therapeutic effect of specific immunoglobulins Y on clinical and subclinical mastitides, diagnosed on the basis of symptomatology, milk analysis and bacterial examination. The microbiological testing of the samples harvested upon completion of the treatment yielded negative results for all the initially identified pathogens. Laboratory examinations showed significant decrease of the somatic cell counts. Besides restoring the required milk quality, there no problem with the consumption of milk from the treated cows and no mandatory withdrawal period exist (as is the case for antibiotics).

Chapter VII, “*General conclusions and recomandations*” addresses the conclusions that can be drawn following the testing of specific immunoglobulins Y in the amelioration and control of certain diseases in humans and animals, especially in the context of explosion of the antibiotic resistance phenomenon at planetary level. As a recommendation, due to the exceptional results obtained with the IgY containing products, both physicians and patients, must be made aware of the immense potential of immunoglobulins Y in the treatment/alleviation/prevention of various acute/chronic conditions.