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HUMAN ADA2-SPECIFIC IgY ANTIBODIES FROM HEN EGGS WITH TOLERANCE TO HUMAN IgG

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Purification of human plasma adenosine deaminase 2 (ADA2) is challenging due to its low physiological concentration and co-purification of human immunoglobulin G (IgG), which has similar physicochemical properties. This study describes a novel approach exploiting avian immune tolerance to human IgG to selectively produce antibodies against native and active human ADA2. Chickens were rendered tolerant to human IgG, enabling subsequent immunization with a preparation containing both ADA2 and human IgG. This strategy successfully elicited a specific immune response, yielding antibodies exclusively to ADA2. These anti-ADA2 antibodies (IgY), purified from egg yolks, were subsequently employed in an affinity column. This immunopurification method allowed the isolation of an electrophoretically homogeneous preparation of human ADA2. This study demonstrates the utility of inducing immune tolerance to human IgG in chickens.

K e y w o r d s: egg yolk immunoglobulin Y; human adenosine deaminase 2; human IgG; immune tolerance; oriented immobilization; affine purification.

Immune tolerance is the absence of a specific immune response to a particular antigen, including those encountered in allogeneic transplants. Building on the theoretical framework established by Burnet F.M. and Fenner F. regarding immune self-recognition, Medawar P. et al. experimentally demonstrated that potential antigens reaching lymphoid cells during the prenatal period can suppress future immune responses. This phenomenon was termed ‘acquired immune tolerance’. Medawar P. et al.’s work confirmed that the immune system learns to distinguish between ‘self’ and ‘non-self’ during early development, leading to specific unresponsiveness to antigens encountered during this critical time [1, 2]. Immune tolerance can be induced artificially through various methods, some of which have medical applications, such as preventing organ transplant rejection and treating autoimmune and allergic diseases [3, 4].

Inducing immune tolerance in chickens, a valuable model for immunological studies, was achieved by inoculating the antigen early in embryonic development [5-7]. Zhao C. et al. compared two methods of antigen introduction to chicken embryos: microinjection into the blood vessel or direct injection into the egg yolk. They found that the latter method is more suitable for obtaining tolerant chickens, as it is more convenient and results in a higher hatch rate [8].

We employed this method of injecting antigens into eggs to induce immune tolerance in chickens, aiming to generate antibodies against ADA2, a human blood plasma enzyme.

ADA2 is one of two isoenzymes of human adenosine deaminase (ADA, EC 3.5.4.4), a crucial enzyme in purine metabolism. It plays a vital role in regulating the levels of (deoxy)adenosine in animal and human tissues, converting them into (deoxy) inosine. The two isoenzymes, ADA1 and ADA2, are encoded by different genes and vary in their catalytic activity and function. ADA1, which has been more extensively studied (with a K_m of approximately 0.04 mM and a molecular weight of 35-41 kDa), is encoded by a gene located on human chromosome 20q.11.33. In contrast, ADA2 (with a K_m of around 2.0 mM and a molecular weight of 110-120 kDa), encoded by a gene on chromosome 22q11.1 (known as CECR1), is less well characterized. Both ADA isoenzymes are significant in regulating adenosine concentration and are involved in the development of the

immune system [9]. While ADA1 is predominantly an intracellular enzyme vital for a healthy immune system – primarily through its role in purine metabolism and preventing the toxic accumulation of deoxyadenosine in lymphocytes, and whose deficiency leads to lymphotoxicity and a dramatic reduction in T- and B-lymphocyte numbers – ADA2 is a less well-studied isoenzyme due to its low expression in mammalian tissues. In humans, ADA2 is primarily found in blood plasma, exhibiting low activity under normal conditions, but increasing significantly in conditions such as immune disorders, hypoxia, inflammation, and various infections [10-12].

Patients with ADA2 deficiency (DADA2) experience health problems, including early-onset inflammation, strokes, vasculitis, and bone marrow failure [13, 14]. Changes in immune cells suggest ADA2's role in immune system balance; its unique function is to induce the differentiation of monocytes into macrophages/dendritic cells [15]. ADA2 has recently been discovered in lysosomes, where can regulate adenosine levels and influence cell activation, particularly the polarization of macrophages into M1/M2 phenotypes [16, 17].

Given the diagnostic importance of adenosine deaminase (ADA) activity, particularly in tuberculous pleurisy, meningitis, and related conditions, colorimetric methods are the primary means of analysis [10-12]. However, antibodies specific to ADA isoenzymes offer a valuable complementary diagnostic approach due to their high specificity and sensitivity [18, 19]. While additional, more extensive purification steps (e.g., multiple chromatography or alternative methods like epitope-specific screening or monoclonal antibodies) could further reduce IgG contamination, they would significantly increase the complexity, time, and cost of antigen preparation, especially given the 'negligible amounts' of ADA2 in plasma. Our method for isolating ADA2, which still contains IgG contamination, is available and inexpensive.

Łupicka-Słowik A. et al. developed avian IgY specific to the ADA1 isoenzyme [20].

This work proposes another approach to developing ADA2-specific antibodies in immunized chickens, by employing the principle of immune tolerance to specifically target ADA2.

Our primary goal was to induce immune tolerance to human IgG in chickens. This strategy was crucial because it allowed us to immunize the chickens with an ADA2 preparation containing human IgG as a contaminant. By establishing tolerance, we aimed to generate antibodies specifically against native ADA2, minimizing cross-reactivity with human IgG, a major in purification hurdle.

Materials and Methods

Donor human blood plasma was provided by the R. O. Yeolian Haematology Centre. Fertilized eggs of the 'Yerevan Red' chicken breed were sourced from a local poultry farm. This breed was selected for its widespread use in egg production and immunological research, known robustness, low maintenance requirements, and natural high resistance to various infections and extreme environmental conditions.

The following reagents were used in this study: DEAE-Sephadex A-50, CM-Sephadex C-50, SP-Sephadex C-50, and Sepharose CL6B (Pharmacia, Sweden); adenosine, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), and sodium m-periodate (Sigma Chemical Co., St. Louis, MO, USA); and polyethylene glycol M 6000 (PEG 6000) (Merck, Germany). All other reagents were of high purity.

Preparation and purification of antigens (ADA2 and IgG) from donor plasma: The isolation and purification of ADA2 was performed as previously described [21]. Briefly, 200 ml of donor plasma was dialyzed against 0.01 M potassium phosphate buffer (pH 7.4), diluted 1:2, and then applied to a DEAE-Sephadex column (2.5 cm × 8.0 ml), pre-equilibrated with the same buffer. The resulting eluate, depleted of ADA1 and other serum proteins, was adjusted to pH 6.0–6.2 and loaded onto a CM-Sephadex column (2.5 cm × 8.0 ml). Gradient elution, using 0.01 M potassium phosphate buffer containing 0.08–0.40 M potassium chloride, yielded distinct IgG and ADA2 fractions. ADA2 activity was determined, and the absence of ADA1 activity was confirmed. Both the ADA2 and IgG fractions were then separately purified by further chromatography on an SP-Sephadex column.

ADA2 activity was determined by measuring the amount (μmol) of ammonia produced per milligram of protein per minute. Protein fractions were concentrated using an Amicon-50 ultrafiltration device (Amicon Ultra 50K, USA) and analyzed by 7.5% polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS-PAGE) [22].

The IgG fraction, after its second chromatography step on the SP-Sephadex column, was used

for yolk sac inoculation. ADA2 fractions that contained IgG contamination were specifically used for chicken immunization.

Protein concentration was determined using two methods: the Bradford assay [23], with bovine serum albumin (BSA; Sigma, USA) as a standard, and spectrophotometry at 280 nm, employing a protein extinction coefficient of 1.02 $(mg/ml)^{-1} \cdot cm^{-1}$.

Incubation and hatching of experimental chickens: a cohort of 24 fertilized chicken eggs was used for experimental purposes. Following yolk sac injection, 11 chicks successfully hatched (5 cockerels and 6 pullets), resulting in an approximate hatch rate of 50%. Due to the practical challenges of early chick sexing and the specific requirement for IgY production from laying hens, the sex distribution of newly hatched chicks was not controlled prior to the identification of mature hens selected for IgY production. All eleven hatched chicks completed the IgG immunization protocol and served as biological replicates.

Eggs were incubated at 38.5°C with 70% relative humidity and automatically rocked through a 90° angle every two hour. Newly hatched chicks were housed in a separate room at 30°C for the first week post-hatch, subsequently maintained at 20°C. All chicks had ad libitum access to water and feed throughout the study.

Induction of immune tolerance to human IgG. Immune tolerance to human IgG was induced via yolk sac inoculation. Microinjection was performed on embryonic days 5–7, identified as the optimal period for establishing tolerance [8]. A 0.1 ml solution containing 0.1 mg of human IgG (in 0.01 M sodium phosphate buffer, pH 7.0, with 0.15 M sodium chloride) was vertically injected into the air cell at the blunt end of each egg using a syringe. The puncture hole was then sealed with paraffin wax, and eggs were returned to the incubator until hatching.

Post-hatch immunization to induce tolerance. At three weeks of age, chickens received a primary subcutaneous injection across multiple sites. Each injection comprised 0.2 ml of human IgG solution (0.2 mg protein), thoroughly mixed with an equal volume of FCA. Booster immunizations were administered on days 10 and 20 following the primary injection, each consisting of the same amount of human IgG emulsified with an equal volume of FIA. Ten days after the final adjuvant-assisted injection, the same doses of human IgG were administered intramuscularly without adjuvant. At this stage, the six female chickens were selected for further immunization due to their ability to produce IgY in eggs.

Immunization with ADA2 preparation. At seven months of age, the selected female chickens were immunized with an ADA2 preparation (which contained human IgG). The initial inoculation used FCA, followed by a booster immunization with FIA one month later.

Assessment of immune response. Blood samples (0.5–1.0 ml) were collected from a wing vein of chickens at three weeks and two months post-immunization with human IgG, and again one month after the second immunization with the ADA2 preparation. Samples were stored for 24 h at 4°C, after which serum was separated by centrifugation at 568 ×g for 10 min.

To assess tolerance, collected serum samples were tested for precipitating antibodies against human IgG using the agarose gel double diffusion method [24]. Following immunization with the ADA2 preparation, hen blood serum was similarly analyzed for precipitation with both human IgG and the ADA2 preparation. All serum samples were tested in duplicate.

Isolation and purification of IgY from egg yolks. Following the detection of specific anti-ADA2 antibodies, eggs were collected daily, labelled, and stored at 4°C. IgY was isolated from egg yolks using PEG 6000 and ion-exchange chromatography on DEAE-Sephadex, as detailed in [25]. The yield of purified IgY from a single egg yolk was approximately 50–70 mg.

Preparation of an affinity column with oriented IgY. An affinity column with oriented IgY was prepared. IgY was directionally immobilized onto Sepharose CL6B using sodium m-periodate oxidation, as described by [26].

Affinity purification procedure. The resulting IgY-Sepharose CL6B affinity column (2 ml), prepared in phosphate-buffered saline (0.15 M NaCl and 0.02 M potassium-phosphate buffer, pH 7.4), was used to purify the ADA2 preparation. Briefly, an ADA2 sample containing IgG (total protein: 1.5 mg/ml) was passed through the column. After washing the column with phosphate-buffered saline (0.15 M NaCl and 0.02 M potassium-phosphate buffer, pH 7.4) to remove non-specific proteins, the ADA2 was eluted from the column with 0.2 M glycine-HCl buffer (pH 2.6). The pH of the eluted protein solution was immediately adjusted to 6.0 using glycine-NaOH buffer (pH 8.6). Subsequently, the ADA2

preparation was dialyzed against phosphate-saline buffer (pH 6.0), concentrated using an Amicon-50 ultrafiltration device, and analyzed by SDS-PAGE [22].

Spectral measurements were conducted using a Cary 60 spectrophotometer (Agilent, USA) and a Specoll-211 (Germany).

Results and Discussion

To ensure the integrity of the experimental conditions, we first confirmed the purity of the ADA2 and IgG preparations. SDS-PAGE electrophoresis verified that both preparations were free from any pre-mixed components (Fig. 1, *A*).

We observed no serum precipitation with IgG, indicating successful induction of immune tolerance to IgG in all immunized chickens, on figure presented some of them (Fig. 2, *A*). Following this, chickens were immunized with an ADA2 preparation containing IgG. A month post-immunization, blood samples were collected, and isolated serum was tested for precipitation with IgG, ADA1, and ADA2. Crucially, a distinct precipitation pattern was observed specifically with ADA2, confirming the successful generation of ADA2-specific antibodies (Fig. 2, *B*).

After successfully detecting specific antibodies to ADA2 in the hens' serum, we collected eggs from these birds and stored them at 4°C for further processing.

Egg yolk IgY was then extracted and purified using the protocol detailed in [25]. However, the extracted IgY contained significant lipid contaminants from the egg yolk. Since even minor lipid contaminants can induce protein oxidation and denaturation [26], we performed three treatments with an equal volume of chloroform to remove them and ensure the stability and integrity of the IgY.

As shown in Fig. 2, *B*, a clear precipitin line formed between the purified IgY and the ADA2 preparation (which included IgG). Importantly, we observed no precipitation lines between the generated antibodies and human ADA1 or IgG, confirming the specificity of the isolated IgY for ADA2.

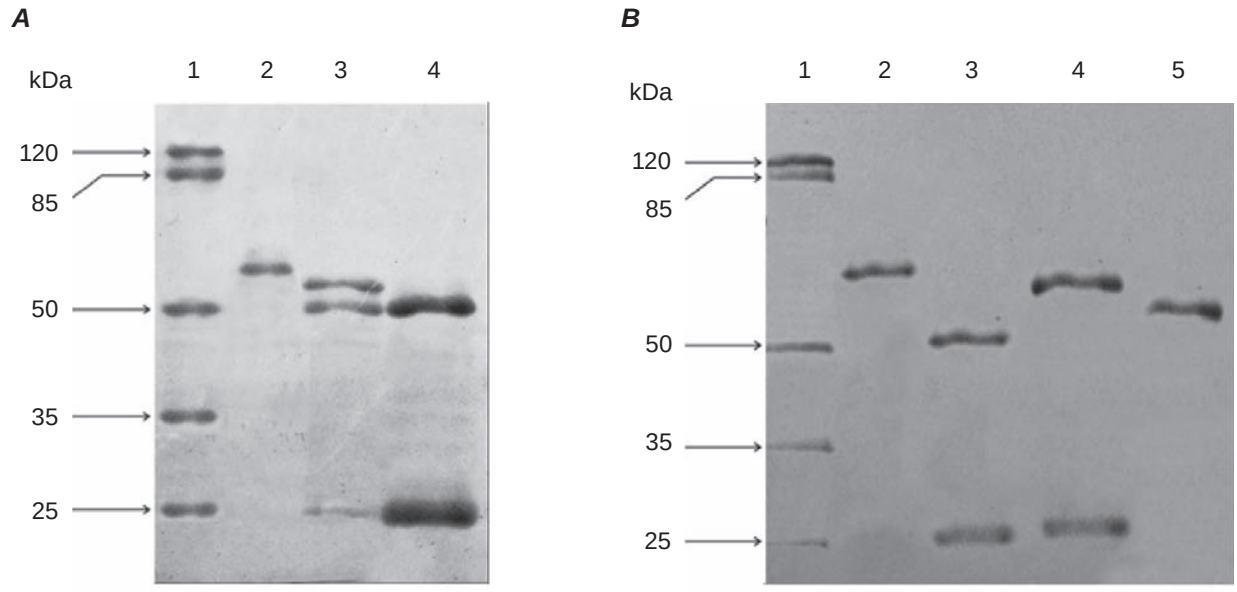

*Fig. 1. Electrophoretic separation of purified ADA2 and IgG preparations used for immunization (**A**); fractions after IgY-Sepharose CL6B affinity chromatography (**B**). **A** – Lane 1: Molecular weight markers (kDa). Lane 2: Bovine serum albumin (BSA) standard (68 kDa, 30 μg loaded). Lane 3: ADA2/IgG preparation (50 μg): The band at approximately 60 kDa corresponds to the monomer of ADA2 under denaturing SDS-PAGE conditions, consistent with the reported size of the monomer of the homodimeric ADA2 (110-120 kDa). Lane 4: Human IgG (IgG): heavy chain (50 kDa) and light chain (25 kDa) (70 μg loaded). **B** – Lane 1: Molecular weight markers (kDa). Lane 2: Bovine serum albumin (BSA) standard (30 μg). Lane 3: Human immunoglobulin G (IgG). Lane 4: Immunoglobulin Y (IgY) purified from egg yolk, used for affinity column preparation: heavy chain (64 kDa) and light chain (27 kDa) (50 μg). Lane 5: ADA2, purified via IgY affinity chromatography and concentrated using an Amicon 50 ultrafiltration unit. (Gels were stained with Coomassie G-250 dye)*

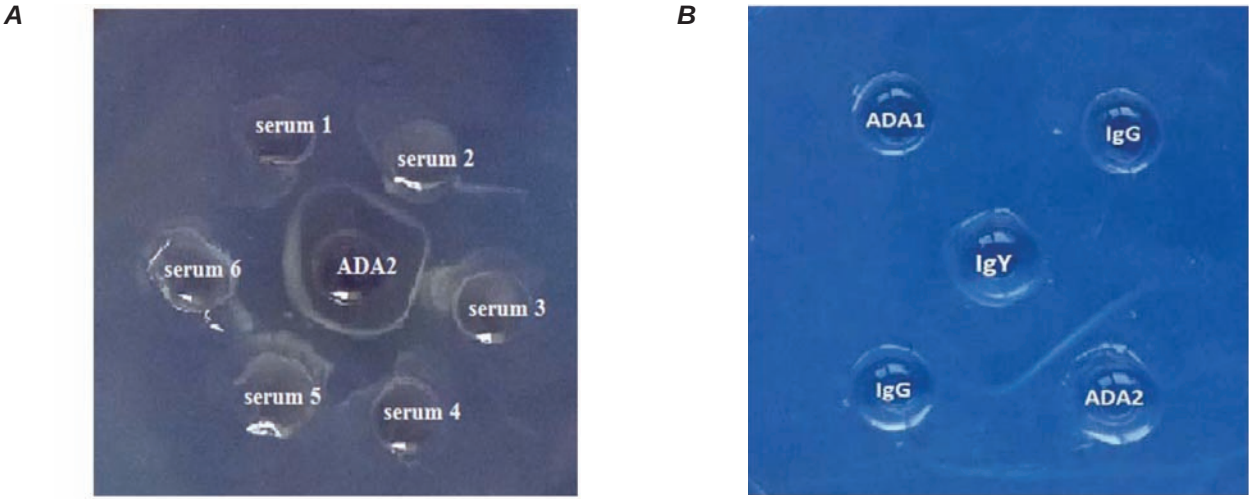

*Fig. 2. Precipitation reactions between chick serum samples (1-6) and ADA2 (containing IgG) (**A**); precipitation reaction between purified chick IgY, human IgG, ADA1 and ADA2 (containing IgG) preparations (**B**)*

Fig. 1, *B* (Lane 4) displays the SDS-PAGE image of IgY. This analysis confirmed the purity of the IgY preparation, showing no discernible contaminants. At this purification stage, the IgY was subsequently used to make an affinity column, ensuring the oriented attachment of the antibodies for optimal binding efficiency.

Fig. 1, *B* (Lane 5) displays the SDS-PAGE pattern of the ADA2 preparation following purification on this newly developed affinity column. The presence of a single, distinct band in the SDS-PAGE confirms the successful production of highly selective antibodies to ADA2 and the effective purification of the target protein. Furthermore, the protein obtained through this affinity purification process, after elution and subsequent dialysis, retained its enzymatic activity, providing strong evidence for the successful isolation of the native and active ADA2 enzyme.

It's well-established that protein aggregation can significantly increase their immunogenicity, mainly because macrophages take them up more efficiently [27]. For such proteins, achieving immune tolerance in experimental animals can be difficult, or even impossible. Conversely, using soluble proteins, such as albumin or immunoglobulin, generally makes it easier to induce a tolerant state [28]. The results of our experiments, in which all eleven hatched chickens showed tolerance to IgG, convincingly confirm this principle. The local breed of 'Yerevan Red' chickens used for the experiment fully met the requirements of the task and are well-suited for immunological studies to obtain antibodies.

Initially, our attempts to prepare an IgY affinity column using standard methods were unsuccessful. Consequently, we adopted a method involving the oriented immobilization of antibodies on Sepharose, which yielded the desired outcome. This successful approach allowed us to produce polyclonal antibodies specifically targeting the native and active ADA2 enzyme. These antibodies represent a valuable tool for future investigations, enabling the purification of homogeneous ADA2 enzyme.

Chicken-derived IgY antibodies are increasingly prominent in research and diagnostics due to several key advantages. Their extraction from egg yolks is simple, ethical, and cost-effective, while maintaining antibody quality. The significant evolutionary distance between birds and mammals enhances the immunogenicity of chicken antibodies, boosting their diagnostic potential. Additionally, IgY antibodies reduce false-positive results in serological assays because they do not interact with rheumatoid factor or complement components [29-30].

Despite initial purification steps (DEAE-Sephadex, CM-Sephadex, and SP-Sephadex chromatography), it was consistently difficult and costly to achieve an ADA2 preparation entirely free of human IgG contamination. This co-purification makes it challenging to raise specific antibodies without also generating an immune response against the highly abundant human IgG.

Obtaining specific antibodies against ADA2 is significantly challenging due to the negligible quantity of this enzyme in human serum. Furthermore, the consistent co-purification of ADA2 with IgG, which shares similar physicochemical properties, complicates the purification process and increases its cost. Standard antibody production methods would likely result in antibodies targeting both isoenzyme ADA2 and IgG. To overcome this limitation and produce ADA2-specific antibodies, we leveraged the principle of immune tolerance.

Conclusions. This study has demonstrated the successful application of a method for inducing immune tolerance to human IgG in young chicks. We leveraged this inherent characteristic of chicks to generate highly specific IgY antibodies in hens targeting the native serum ADA2 isoenzyme.

The purification of this enzyme from blood plasma in sufficient quantities presents a significant challenge. However, our approach offers a practical and cost-effective solution, as the isolation and purification of ADA2, even when initially contaminated solely with IgG, proved accessible and inexpensive. The method for producing IgG-tolerant chicks is also readily achievable. A key advantage of this strategy for obtaining anti-ADA2 antibodies is their utility in purifying homogeneous ADA2 enzyme for basic research without requiring costly materials, and their potential application in diagnostics without interference from IgG.

Utilizing chicks for immunization provides substantial benefits, primarily by eliminating the need for repeated blood collection from laboratory animals.

Furthermore, the simple collection of eggs offers a convenient and abundant source of antibodies. While this study successfully produced specific antibodies, further work is necessary to quantify their precise characteristics, which was a limitation of the current research.

Conflct of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ЛЮДСЬКІ ADA2-СПЕЦИФІЧНІ АНТИТІЛА IGY ІЗ ЯЄЦЬ КУРЕЙ, ТОЛЕРАНТНИХ ДО IGG ЛЮДИНИ

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Очищення аденозиндезамінази 2 з плазми крові людини (ADA2) становить складне завдання через її низьку фізіологічну концентрацію та одночасне ко-очищення імуноглобуліну G людини (IgG), який має подібні фізико-хімічні властивості. У нашому дослідженні представлено новий підхід, що ґрунтується на індукції імунної толерантності курей до IgG людини з метою селективного отримання антитіл проти нативної та активної ADA2 людини. Попереднє формування у курей толерантності до людського IgG дозволило подальшу імунізацію препаратом, який містив як ADA2, так і IgG. Така стратегія сприяла специфічній імунній відповіді з продукцією антитіл виключно проти ADA2. Отримані антитіла до ADA2 (IgY), виділені з яєчного жовтка, надалі піддавалися афінному очищенню. Запропонований метод імуноочищення забезпечив отримання електрофоретично гомогенного препарату людської ADA2. Таким чином, дослідження демонструє доцільність індукції імунної толерантності до людського IgG у курей.

Ключові слова: імуноглобулін Y з жовтка яйця. аденозиндезаміназа 2 людини. IgG людини. імунна толерантність. орієнтована іммобілізація. афінне очищення.

References

1. Brent L. Transplantation tolerance – a historical introduction. *Immunology.* 2016; 147(3): 267-268.
2. Ribatti D. Peter Brian Medawar and the discovery of acquired immunological tolerance. *Immunol Lett.* 2015; 167(2): 63-66.
3. Luo X, Miller SD, Shea LD. Immune Tolerance for Autoimmune Disease and Cell Transplantation. *Annu Rev Biomed Eng.* 2016; 18: 181-205.

4. Akdis CA. Therapies for allergic inflammation: refining strategies to induce tolerance. *Nat Med*. 2012; 18(5): 736-749.
5. Wolfe HR, Tempelis C, Mueller A, Reibel S. Precipitin production in chickens. XVIL The effect of massive injections of bovine serum albumin at hatching on subsequent antibody production. *J Immunol*. 1957; 79(2): 147-153.
6. Stevens KM, Pietryk HC, Ciminera JK. Acquired immunological tolerance to a protein antigen in chickens. *Br J Exp Pathol*. 1958; 39(1): 1-7.
7. Ivanyi J, Hraba T. A contribution to the study of immunological tolerance to protein antigen in the chicken. *Folia Biol (Praha).* 1963; 9: 354-363.
8. Zhao C, Song C, Wang X, Li Z, Sha J, Han H, Zhang Y, Li Z. Induction of immunological tolerance in chickens inoculated with xenogeneic antigens at an early stage of embryonic development. *Dev Comp Immunol.* 2006; 30(4): 431-440.
9. Antonioli L, Colucci R, La Motta C, Tuccori M, Awwad O, Da Settimo F, Blandizzi C, Fornai M. Adenosine deaminase in the modulation of immune system and its potential as a novel target for treatment of inflammatory disorders. *Curr Drug Targets.* 2012; 13(6): 842-862.
10. Palomino JC. Nonconventional and new methods in the diagnosis of tuberculosis: feasibility and applicability in the field. *Eur Respir J*. 2005; 26(2): 339-350.
11. Andreasyan NA, Hairapetian HL, Sargisova YG, Mardanyan SS, Badalyan LT, Khanoyan AS. Activity of adenosine deaminase and its isoforms in pleural fluid in tuberculous pleuritis. *Med Sci Monit*. 2002; 8(10): CR708-CR712.
12. Dos Santos Jaques JA, Becker LV, Souza Vdo C, Leal CA, Bertoldo TM, de Vargas Pinheiro K, Morsch VM, Schetinger MR, Leal DB. Activities of enzymes that hydrolyze adenine nucleotides in lymphocytes from patients with rheumatoid arthritis. *Cell Biochem Funct*. 2013; 31(5): 395-399.
13. Lee PY. Vasculopathy, Immunodeficiency, and Bone Marrow Failure: The Intriguing Syndrome Caused by Deficiency of Adenosine Deaminase 2. *Front Pediatr.* 2018;6:282.
14. Caorsi R, Penco F, Schena F, Gattorno M. Monogenic polyarteritis: the lesson of ADA2 deficiency. *Pediatr Rheumatol Online J*. 2016; 14(1): 51.
15. Zavialov AV, Gracia E, Glaichenhaus N, Franco R, Zavialov AV, Lauvau G. Human adenosine deaminase 2 induces differentiation of monocytes into macrophages and stimulates proliferation of T helper cells and macrophages. *J Leukoc Biol*. 2010; 88(2): 279-290.
16. Greiner-Tollersrud OK, Boehler V, Bartok E, Krausz M, Polyzou A, Schepp J, Seidl M, Olsen JO, Smulski CR, Raieli S, Hübscher K, Trompouki E, Link R, Ebersbach H, Srinivas H, Marchant M, Staab D, Guerini D, Baasch S, Henneke P, Kochs G, Hartmann G, Geiger R, Grimbacher B, Warncke M, Proietti M. ADA2 is a lysosomal DNAse regulating the type-I interferon response. *bioRxiv*. 2020; 2020-06.
17. Greiner-Tollersrud OK, Krausz M, Boehler V, Polyzou A, Seidl M, Spahiu A, Abdullah Z, Andryka-Cegielski K, Dominick FI, Huebscher K, Goschin A, Smulski CR, Trompouki E, Link R, Ebersbach H, Srinivas H, Marchant M, Sogkas G, Staab D, Vågbø C, Guerini D, Baasch S, Latz E, Hartmann G, Henneke P, Geiger R, Peng XP, Grimbacher B, Bartok E, Alseth I, Warncke M, Proietti M. ADA2 is a lysosomal deoxyadenosine deaminase acting on DNA involved in regulating TLR9-mediated immune sensing of DNA. *Cell Rep*. 2024; 43(11): 114899.
18. Kim SH, Park MK, Kim JY, Chuong PD, Lee YS, Yoon BS, Hwang KK, Lim YK. Development of a sandwich ELISA for the detection of Listeria spp. using specific flagella antibodies. *J Vet Sci*. 2005; 6(1): 41-46.
19. Brujeni GN, Gharibi D. Development of DNA-designed avian IgY antibodies for detection of *Mycobacterium avium* subsp. paratuberculosis heat shock protein 70 (Hsp70) and anti-Hsp70 antibodies in the serum of normal cattle. *Appl Biochem Biotechnol*. 2012; 167(1): 14-23.
20. Łupicka-Słowik A, Psurski M, Grzywa R, Bobrek K, Smok P, Walczak M, Gaweł A, Stefaniak T, Oleksyszyn J, Sieńczyk M. Development of Adenosine Deaminase-Specific IgY Antibodies: Diagnostic and Inhibitory Application. *Appl Biochem Biotechnol*. 2018; 184(4): 1358-1374.
21. Andreasyan NA, Hairapetyan HL, Sargisova YG, Mardanyan SS. ADA2 isoform of adenosine deaminase from pleural fluid. *FEBS Lett*. 2005; 579(3): 643-647.

22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 1970; 227(5259): 680-685.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72(1-2): 248-254.
24. Ouchterlony O. Diffusion-in-gel methods for immunological analysis. *Prog Allergy*. 1958; 5: 1-78.
25. Polson A, Coetzer T, Kruger J, von Maltzahn E, van der Merwe KJ. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. *Immunol Invest*. 1985; 14(4): 323-327.
26. Yeritsyan HE, Gasparyan VK. Homogeneous immunoassay for human IgG using oriented hen egg IgY immobilized on gold sol nanoparticles. *Microchim Acta*. 2012; 176: 117-122.
27. Pham NB, Meng WS. Protein aggregation and immunogenicity of biotherapeutics. *Int J Pharm*. 2020; 585: 119523.
28. Lundahl MLE, Fogli S, Colavita PE, Scanlan EM. Aggregation of protein therapeutics enhances their immunogenicity: causes and mitigation strategies. *RSC Chem Biol*. 2021; 2(4): 1004-1020.
29. Lee L, Samardzic K, Wallach M, Frumkin LR, Mochly-Rosen D. Immunoglobulin Y for Potential Diagnostic and Therapeutic Applications in Infectious Diseases. *Front Immunol*. 2021; 12: 696003.
30. Vieira-Pires RS, Morgan PM, Choraria A, Gandhimathi C, Michael A, Ôchoa-Pires T, Salimian J, Rajan S, Subramani M. Applications of IgY in Human Medicine. In: Zhang XY, Vieira-Pires RS, Morgan PM, Schade R. (eds) IgY-Technology: Production and Application of Egg Yolk Antibodies. Springer, Cham., 2021: 237-278.