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The preparation of N-IgY targeting SARS-CoV-2 and its immunomodulation to IFN-γ production in vitro

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A B S T R A C T
Specific antibodies against SARS-CoV-2 structural protein have a wide range of effects in the diagnose, prevention and treatment of the COVID-19 epidemic. Among them, egg yolk immunoglobulin Y (IgY), which has high safety, high yield, and without inducing antibody-dependent enhancement, is an important biological candidate. In this study, specific IgY against the conservative nucleocapsid protein (NP) of SARS-CoV-2 was obtained by immunizing hens. Through a series of optimized precipitation and ultrafiltration extraction schemes, its purity was increased to 98%. The hyperimmune IgY against NP (N-IgY) at a titer of 1:50,000 showed strong NP binding ability, which laid the foundation of N-IgY’s application targeting NP. In an in vitro immunomodulatory study, N-IgY (1 mg/mL) modulated NP-induced immune response by alleviating type II interferon (IFN-γ) secretion stimulated by NP (20 μg/mL). In summary, N-IgY can be mass produced by achievable method, which endows it with potential value against the current COVID-19 pandemic.

1. Introduction

It has been fifteen months since the outbreak of the Coronavirus Disease 2019 (COVID-19) in early 2020 [1]. However, the rampant severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the pathogen causing COVID-19, has not stopped. Although the epidemic slowed down last summer under the current situation where the public were becoming more and more familiar with the phenomenon of “not wearing a mask”, it soon began to worsen in autumn and winter. Society had always placed high hopes on the role of vaccines. Indeed, after the start of the promotion of SARS-CoV-2 vaccines, the number of confirmed cases worldwide per week began to decline since the beginning of this year. But the good times didn’t last long. Starting from mid-February, the number of confirmed cases increased significantly in all areas with more severe epidemics, with the increased number of deaths [2]. In this confusing situation, the “vaccine passport” or “immunity passport” policy had been implemented in some countries and regions under the premise that there were still widespread disputes about its ethics consideration and the effectiveness for epidemic control [3–4].

The pressure of economic development and the desire for freedom made strict isolation measures increasingly difficult to implement and led to an unclear trend of pandemic.

The main structural proteins of coronavirus particles are spike (S) protein, membrane (M) protein, envelope (E) protein and nucleocapsid (N) protein [5]. The gene mutation of S protein is one of the current thorny problems in COVID-19 epidemic [6]. While the more conservative N protein (NP) has always been a hot target in diagnosis [7–9] and treatment [10]. Apart from its role in antigen detection, NP can induce specific antibodies in the early stages of the disease course because of its strong antigenicity, which makes it prominent in serological testing as well [11–13]. NP can also be used as a therapeutic target, although it is not a conventional vaccine component candidate [14].

Specific, high-yield antibodies are the prerequisite for the implementation of therapeutic and diagnostic application. In view of the cost-effectiveness, immunoglobulin Y (IgY) may play a certain role in the battle against COVID-19, which had attracted researchers’ attention [15–17]. This predominant serum immunoglobulins in birds, reptiles, and amphibians can transfer from the serum of females to the egg yolk,
where they offer passive immunity to embryos and neonates [18–19]. When used as a therapeutic antibody, IgY that can be taken orally does not react with the human complement system or Fc receptor like mammalian immunoglobulin G (IgG) does [20], which alleviate the risk of antibody-dependent enhancement (ADE) [21–23]. In terms of diagnosis, especially in the diagnosis and application of coronavirus, IgY also has a certain research foundation [24–25]. Here, we introduced the production process and effect identification of the purified hyperimmune IgY specifically against the NP of SARS-CoV-2 (called N-IgY). In this study, N-IgY with high purity can specifically bind to NP. Although it was not neutralizing antibody, its blocking ability to NP allows N-IgY to play a role in therapy. We also verified this immunomodulatory effect through an in vitro experiment in this study.

2. Materials and methods

2.1. Immunization of hens

The project was approved by the ethics committee of West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2020-394). A total of nine 16-week-old laying hens were purchased from the Experimental Animal Center of West China School of Basic Medical Sciences & Forensic Medicine, Sichuan University and raised in a clean, ventilated environment.

The NP used to immunize animals was purified recombinant N-His protein, which was synthesized by Guangdong Laboratory Animals Monitoring Institute and Guangdong Provincial Key Laboratory of Laboratory Animals (Guangzhou, China). It was expressed in Escherichia coli by constructing a codon optimized pET28a-N plasmid. After identification by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE), it was stored at –20 °C until use.

The purified NP was adjusted to a concentration of 1 mg/mL with phosphate-buffered saline (PBS), mixed with Freund’s adjuvant (Sigma, USA) or Freund’s incomplete adjuvant in equal proportions and fully emulsified. These two mixtures were used to inject NP-immunized group (group N, n = 6) for the primary or booster immunization. The injection sites were located in the subcutaneous tissues of both wings, and the injection amount of NP for each hen was 500 μg. Three booster immunizations were performed at the end of 2nd, 4th, and 6th week. The control group (group Con, n = 3) was injected with an equal volume of PBS and adjuvant emulsified mixture.

Eggs were collected and stored at 4 °C until subsequent testing. In order to compare the titer of immunoglobulin in yolk and serum, peripheral blood of group N and group Con was harvested from the wing vein one week after the third booster immunization. Sera were separated by centrifugation at 3000 rpm for 10 min at room temperature and stored at –20 °C until subsequent testing.

2.2. Biochemical examination of immunized hens

The immunized hens were euthanized at the end of this study. Non-anticoagulant blood was collected. Sera were separated by centrifugation at 3000 rpm for 10 min at room temperature and then analyzed by automatic biochemical analyzer (AU680, Beckman, USA) for twenty-eight routine biochemical indicators.

2.3. Purification of IgY

Based on Akita and Nakai’s research on precipitation of IgY from WSF with ammonium sulfate (AS) [26], we tried different adjustments. At the beginning, water-soluble fraction (WSF) was extracted with water dilution method. After summarizing the various options in different studies [26–32], we adopted two types of extraction schemes to precipitate crude IgY from WSF. Please refer to the supplementary materials for screening protocol in detail. The molecular weight of IgY composed of two light chains and two heavy chains is about 180 kDa (kDa) [22,43], so the centrifugal filter units with the pore size of 100 kDa (Amicon Ultra, Millipore, USA) were used finally for desalination and concentration. The supernatants obtained by ultrafiltration was the final purified IgYs. They could be stored at –20 °C directly after the concentration was determined or stored at 4 °C after lyophilization.

2.4. Determination of IgY titer

The determination of antibody titer and the preparation of corresponding reagents are based on the enzyme-linked immunosorbent assay (ELISA) introduced in the similar studies [25,33] with a few adjustments. The coating concentration of NP and the dilution ratio of IgY and horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgY secondary antibody (Bios, China) was determined according to the checkerboard method. Each well in the polystyrene high bind strip well microplate (Corning, USA) was coated with 100 μL of NP diluted to 2 μg/mL with carbonate buffer solution (CBS). The plate was incubated overnight at 4 °C. Each well was blocked with bovine serum albumin (BSA) after three times of washing. Then the plate was placed in a wet box and incubated for 2 h at 37 °C. After washing and drying, the plate was stored at 4 °C for further use.

In order to detect and analyze the IgY titer fluctuations of eggs laid after different weeks of primary immunization, WSF was diluted 400 times and added to the NP-coated wells (100 μL/well, with two repeats) respectively. After 1 h of incubation at 37 °C and three times of washing, HRP-conjugated rabbit anti-chicken IgY secondary antibody was added to each well (1:3000, 100 μL/well). After an incubation at 37 °C for 30 min and three times of washing, 100 μL of tetramethylbenzidine (TMB) (Solarbio, China) was added to each well. The color developing reaction time was 15 min, and then equal volume of 1 M hydrochloric acid (HCL) was added to stop the reaction. The background-normalized absorbance value at 450 nm (OD450nm) was determined by microplate reader (SpectraMax iD3, Austria). The OD value was the basis for selection of hyperimmune IgY against NP (called N-IgY).

According to the final optimal purification scheme, N-IgY was obtained. Its titer was determined at the dilution rates of 1:400, 1:2000, 1:10000 and 1:50,000. As the titer of N-IgY might increase significantly compared to WSF, the dilution of the secondary antibody for this titer detection was adjusted to 1:6000. Except for the dilution rate, other operations remained unchanged.

In addition, we compared the titers between N-IgY from yolk and immunoglobulin against NP in serum according to the operations described above.

2.5. Identification of N-IgY and its binding ability to NP

In order to determine the purity of crude IgY obtained by different schemes and the N-IgY, reduced SDS-PAGE was used for verification. After adjusting WSF and crude IgY to the same concentration, they were reduced with SDS loading buffer before electrophoresis. Based on literature review, the reduced IgY has two main bands, the heavy chain around 67 kDa and the light chain around 23 kDa. Therefore, the reduced samples can be separated clearly by 10 ~ 15% polyacrylamide gels.

After the electrophoresis was complete, the gel was stained with Coomassie Brilliant Blue R-250 (Biosharp, China) or transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-rad, USA) using a membrane transfer system (Bio-rad, USA) for further Western blot assay as described in next paragraph. The membrane containing WSF and crude IgY was directly started with secondary antibody incubation, and the subsequent operations were the same. The polycrylamide gels were exposed by an automatic exposure system (ChemilDoc™, Bio-rad, USA) after destaining. By comparing the bands on gel and on PVDF membrane, non-target bands could be clearly distinguished since they would not be bound by secondary antibody. Image J software was used for grayscale calculation in order to determine the purity of IgY.
For Western blot assay, the PVDF membranes containing NP (N-PVDF) following the same SDS-PAGE operation were firstly blocked with 5% skimmed milk for 1 h. The N-PVDF and N-IgY with a diluted concentration of 8 mg/mL, which was the primary antibody, were incubated at room temperature for 1 h and then placed at 4 °C overnight. The IgY extracted from group Con of hyperimmune period and the IgY extracted from eggs laid before immunized (W0) were also used as the primary antibody to incubate with N-PVDF, in order to identify the specific antigen–antibody binding. The washed N-PVDF were incubated respectively with HRP-conjugated rabbit anti-chicken secondary antibody (1:6000) for 1 h at room temperature. After thorough washing, the PVDF membranes were developed by electrochemiluminescence (ECL) system (Bio-rad, USA).

2.6. In vitro experiment of N-IgY on NP-mediated immune response

The project of this in vitro experiment was approved by the ethics committee of West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2020-355). Samples of anticoagulated peripheral blood were donated by healthy human donors (n = 7). Basic characteristics of the donors were listed in Tab. S1. Peripheral blood mononuclear cells (PBMCs) were separated by Ficoll reagent (Haoyang, China) under density gradient centrifugation according to the operating manual. The induction of interferon-γ (IFN-γ) was then measured to identify the blocking ability of N-IgY.

The isolated PBMCs were incubated in RPMI 1640 culture media (Corning, USA) supplemented with 10% of FBS (Corning, USA). The cells were seeded (5 × 10^5 cells/500 μL/well) in 24-well plate (Corning, USA) and cultured at 37 °C in a 5% humidified CO_2_ atmosphere.

Cells were treated with NP (20 μg/mL) alone (group N), or NP pre-mixed with N-IgY (1 mg/mL) for 30 min (group N + IgY) for 18 h. Those treated with PBS were set as control (group Con). After centrifugation at 4000 rpm and 4 °C for 3 min, the separated supernatant was immediately frozen at −20 °C until detected. Human IFN-γ ELISA kit (MULTI SCIENCES, China) was used to detect the secretion of IFN-γ in the culture supernatant according to the operating manual.

2.7. Statistical analysis

GraphPad Prism software 8.3 was used for statistical analysis. Data were presented as mean ± standard deviation (SD). Student’s t-test or Mann-Whitney U test was used to compare differences between two groups. One-way ANOVA was used for multiple comparison. A value of p < 0.05 was considered to be statistically significant. For titer identification, group N was considered to be “positive” when the OD_450nm_ value was 2.1 times or more of group Con.

3. Results

3.1. NP did not seriously affect the physiological function of hens.

After calculating the average egg production per chicken per week, it was found that the egg production of all hens decreased gradually after 1st booster immunization (Fig. 1B). Laying rate in group Con dropped to the lowest in W5, and recovered rapidly from W6, while the recovery of group N was a little slower. This decrease of laying rate recurred after the 3rd booster immunization at a minor degree in both groups.

In terms of serologic examination, most biochemical indicators did not change significantly because of NP immunization. Only five out of twenty-eight indicators were statistically different between group Con and group N (Fig. S1). These indicators were serum total protein (TP), albumin (ALB), alanine aminotransferase (ALT), serum creatinine (CREA) and urea (UREA) (Fig. 1C). The slight decrease in urea was consistent with the change in serum total protein, which indicated that NP leads to malnutrition to a certain extent. Neither liver nor kidney function was significantly affected.

Fig. 1. NP did not seriously affect the physiological function of hens. (A) Immunization regimen. Each hen in group N was immunized with 500 μg of NP for primary immunization (n = 6). Three booster immunization with 500 μg of NP were performed 14, 28 and 42 days later. An equivalent volume of PBS was used instead to immunize control hens (n = 3). Peripheral blood of each hen was harvest one week or four weeks after the 3rd booster immunization. (B) The fluctuation of the average weekly eggs production of hens in two groups. (C) Biochemical indicators with significant changes of hens at the end of the experiment. Line in each scatter plot represented median. * p < 0.05.
3.2. The optimal precipitation and ultrafiltration scheme obtained IgY of high purity.

After comparing the purification effect between a series of screening schemes (please refer to Supplementary Materials), we chose the scheme of adding saturated ammonium sulfate solution into the WSF to make the final saturation of 33% (AS-33%-s). The purity rate of this scheme was as high as 90% (Fig. S2B, lane 1). Prolonging the precipitation time to overnight improved the purification effect better than 2 h (93% vs 86%) (lane 2 and lane 3 in Fig. 2, S2C and S2D). The purification rate reached more than 95% after ultrafiltration (lane 1 in Fig. 2, S2C and S2D), which illustrated the optimal precipitation and ultrafiltration scheme in our study guaranteed high purity IgY for further functional identification and application.

3.3. The titer of N-IgY reached the peak after the 3rd booster immunization

It could be determined from Fig. 3A that the antibody titer of WSF in group N began to rise in the 1st booster immunization period (i.e., W3 and W4), and increased sharply after the 3rd booster immunization (W6). The data of the 5th and 6th weeks were not shown in the analysis of titer fluctuations in Fig. 3A unfortunately. It was resulted from insufficient quantity of eggs as mentioned before. The titer of W7 ~ W10 fluctuated within a small range, and the highest value fell in W9. Multiple comparison by one-way ANOVA showed that there was only a statistical difference between the titers of W9 and W8 (p = 0.0493). The titer of W9 was not statistically different from the titer at the other two time points, as was the titer of W8 (p > 0.05). Combining this with the data of laying rate (Fig. 1B), we determined the W7 ~ W10 as the hyperimmune period. And the hyperimmune IgY of group N purified followed by the optimal scheme was defined as N-IgY in this study.

The N-IgY with a concentration of 15 mg/mL was then used for further titer detection by ELISA. As shown in Fig. 3B, the titer of N-IgY was as high as 1:50,000 according to positive criterion of 2.1 times compared with group Con (p < 0.05). And inhibition effect of N-IgY on the elevated secretion of IFN-γ was observed in all these cells (p < 0.05 between group N and group N + IgY), with the average reduction rate of 52.18%±27.75%.

4. Discussions

As one of the main structural proteins of SARS-CoV-2, NP has relatively high conservation and stability, which is consistent with previous research conclusions on SARS-CoV [34–36]. After being infected with the virus, the body can produce specific antibodies against NP [12–13,37]. Some studies have found that NP induces specific antibodies and T cell responses earlier than S protein, which is more meaningful in early diagnosis [11,13], while some other studies have different results [38–39]. NP is indispensable in the replication of coronavirus due to its assistance on RNA assembly and virus particle release, and antibody against NP is regarded as the marker of the replicative stage of SARS-CoV-2. Researches on NP of mouse hepatitis virus (MHV), a member of coronaviridae, showed that blocking the recruitment of NP strongly inhibited viral infection [10], and monoclonal antibody to NP protected mice against lethal MHV-2 challenge [40].

In recent months, the S protein and some non-structural proteins (NSP) of the virus have undergone genetic sequence mutations [41–47], which has drawn great attention. Mutations in the virus structure has also appeared in the mink-related epidemic and could transmit to human [48–49]. In this regard, research on vaccines or antibodies targeting NP is also meaningful. From these perspectives we prepared and identified specific antibodies against more conservative NP.

Usually after 5 ~ 7 weeks of immunization, the antibody titer can reach a peak [33]. The consistent result was found in our study, the titer of WSF increased significantly two weeks after the booster immunization (i.e., 4th week) and reach a relatively stable platform between 7th ~ 10th week. Booster immunization is very important to maintain high level of antibody titer. In some studies, where immune period lasted months or more than half a year, more booster immunizations were required. Our experiment was ended at the 10th week after the hyperimmune period was determined since we also aimed at whether the hen is affected by NP. Only a small part of serological biochemical indicators of hens were impacted, representing the loss of protein. The slight decrease in urea was consistent with the change in serum total protein, which showed that NP leads to malnutrition to a certain extent. This loss of protein might be related to insufficient protein intake caused by inappetence or increased protein metabolism. In addition, we found a small number of liver enzymes and creatinase, such as alkaline phosphatase and creatine kinase increased (no statistical significance, data not shown), which seemed to be consistent with the changes in clinical patients [39,50]. In general, the boost immunizations of NP were sufficient to produce a large number of specific antibodies and ensured quite an amount to be passed to the offspring without developing typical pathological changes. This is somewhat similar to the characteristics of the current epidemic, that is, the large group of asymptomatic patients. Notably, in the stage of primary immunization, which mimicked the natural infection, the antibody titer did not elevate significantly. And
Fig. 3. The titer of N-IgY was stable at a high level. (A) Fluctuation of IgY titer in WSF after immunization. The grey and black arrows were corresponding to the time points of primary immunization (PI) and booster immunization (BI) respectively. Data was shown as mean ± SD. The titer of W7 was much higher than that of W4. ** p < 0.01 compared with the titer of W4; *** p < 0.001 compared with the titer of W4. (B) Titer of N-IgY. Data was shown as mean ± SD. Group N was considered “positive” when the OD value was 2.1 times or more of group Con. * “positive”. (C) Titer comparison of N-IgY and immunoglobulin in serum collected from hyperimmune period. Data was shown as mean ± SD. Serum in group N was considered “positive” when the OD450nm value was 2.1 times or more of group Con. * “positive”.

Fig. 4. Specific binding ability of N-IgY to NP. Lane 1, 3, 5, 7: molecular marker (M) exposed by stain-free blot and merged with ECL exposed images. Lane 2: NP identified by SDS-PAGE. Lane 4, 6, 8: N-IgY, IgY of group Con and IgY of W0 bound to the PVDF membrane containing NP as the primary antibodies respectively in Western blot assay. Lane 4: obvious protein band was developed at the position of 55 kDa corresponding to the molecular weight of NP after being incubated with the HRP-secondary antibody and exposed by ECL. Lane 6 and 8: vague band was identified at the position of 55 kDa following the same operations.

Fig. 5. N-IgY inhibited IFN-γ secretion stimulated by NP in PBMCs. Group N: PBMCs were treated with NP (20 μg/mL) alone for 18 h. Group N + IgY: NP was premixed with N-IgY (1 mg/mL) for 30 min and then incubated with PBMCs for 18 h. Group Con: PBMCs were treated with PBS as control for 18 h. IFN-γ in the culture supernatant was measured by ELISA.
immune stimulation and immune regulation functions. In the innate immune response, IFN-γ is secreted by natural killer cells and natural killer T cells. It was one of the representative cytokines of T cell response in SARS-CoV infected persons [71]. Previous studies had worried that interferons might increase the risk of SARS-CoV-2 infection by upregulating the expression of angiotensin-converting enzyme 2 (ACE2) in host cells. Regarding the “double-edged sword” role of interferons in the process of SARS-CoV-2 infection of the host, a dialectical research revealed that the antiviral effects of interferons was able to counteract the risk related to ACE2 induction. But IFN-γ was indeed the member with the lowest antiviral ability and the highest ACE2 induction among all interferons [72]. Meanwhile, “Cytokine storm” or “Cytokine release syndrome” was widely mentioned immune event in this epidemic. A large number of inflammatory cytokines accumulate in severe COVID-19 patients, the most representative one is interleukin-6 (IL-6), and IFN-γ is also an important member. “Cytokine storm” is a key link that leads to aggravation in a variety of immune diseases [73]. Therefore, specific antibody therapy for specific cytokines is one of the feasible methods. Currently the most well-known is Tocilizumab, which targets interleukin-6 receptor [74]. Similarly, “Emapalumab” which is a monoclonal antibody targeting IFN-γ, is also one of the potential treatments for primary hemophagocytic lymphohistiocytosis (HLH) [75]. Recent study found that only the combination of TNF-α and IFN-γ induced inflammatory cell death, and neutralizing antibodies targeting TNF-α and IFN-γ protected mice from mortality and cytokine shock during SARS-CoV-2 infection [76]. On the premise that NP has a strong effect of inducing IFN-γ, it can be speculated that N-IgY has a potential role in blocking the immunogenicity of NP and regulating the secretion of excess cytokines. Our results may provide an idea in case that regulating the expression of IFN-γ is considered during the anti-SARS-CoV-2 immunotherapy. We will try to explore the role of N-IgY in infected and even critical patients in the future.

Before this COVID-19 epidemic, due to the advantages of IgY, it was extensively studied in the treatment of various respiratory viruses [31]. IgY has also made some achievements in the treatment of pathogenic coronaviruses [67]. As early as in 2003 after the SARS epidemic, there was a research on IgY against SARS-CoV, and the immunogen was used to inactivated SARS-CoV [60]. The N-IgY with high specificity and high purity in this experiment could target the NP of SARS-CoV-2 in vitro, which would play a potential role in interfering with virus replication and reducing excessive inflammation. The biological safety of IgY that can be administered orally is reliable, which provides convenience for its potential immunomodulatory applications. IgY does not react with the human complement system or Fc receptor like IgG derived from mammals does, thereby reducing the risk of antibody-dependent enhancement (ADE) [21–23]. As an antibody production method that meets the 3R (Reduce, Reuse, Recycle) principles of animal welfare and has low cost, high yield, and high stability, N-IgY might have potential value for the current COVID-19 epidemic.

CRediT authorship contribution statement

Jingli Lyu: Investigation, Methodology, Formal analysis, Data curation, Visualization, Writing - original draft. Lirong Bao: Investigation, Methodology, Data curation. Xin Shen: Investigation, Methodology, Software. CaiXia Yan: Investigation, Validation. Cheng Zhang: Investigation, Methodology. Wei Wei: Investigation and Formal analysis. Yutao Yang: Investigation, Jia Li: Investigation, Methodology. Jiajia Dong: Formal analysis, Resources. Lijing Xiao: Project administration, Resources. Xuedong Zhou: Conceptualization, Funding acquisition, Supervision. Yan Li: Conceptualization, Methodology, Supervision, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2021.107797.

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