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Article

Norwegian Platform for Production of Antiviral Chicken Egg Yolk Immunoglobulins for Viral Pandemics Preparedness

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Abstract: *Background:* Some viruses cause outbreaks which require immediate attention. Neutralizing antibodies could be developed for viral outbreak management. However, development of monoclonal antibodies is often long, laborious, and unprofitable. Here we report a Norwegian platform for development of chicken polyclonal neutralizing antibodies with powerful therapeutic potential. *Methods:* Layers were immunized twice with 14-day interval using purified RBD of SARS-CoV-2. Eggs were harvested 14 days after the second immunization. Polyclonal IgY antibodies were extracted. Binding of anti-RBD IgY to RBD was measured by indirect ELISA. Neutralization capacity of anti-RBD IgYs was measured in Vero-E6 cells infected with SARS-CoV-2-mCherry strain using fluorescence and cell viability assay. In addition, the effect of IgYs on the expression of SARS-CoV-2 and host cytokine genes in lungs of Syrian Golden hamsters was examined using qRT-PCR. *Results:* Anti-RBD IgYs efficiently bind RBD of S protein of SARS-CoV-2 in situ, neutralize the virus in vitro, and lower viral RNA amplification without significant alteration of virus-mediated immune gene expression in vivo. *Conclusions:* Altogether, our results indicated that chicken polyclonal IgYs can be attractive targets for pre-clinical and clinical development for rapid management of outbreaks of emerging and re-emerging viruses.

Keywords: IgY; SARS-CoV-2; Vero-E6, Syrian Golden hamster; antiviral strategy

1. Introduction

Viral diseases consistently pose a substantial economic and public health burden. This burden is due to the ability of viruses to be transmitted from wild and domestic animals to humans, resulting in unpredictable outbreaks. The current strategy for the viral outbreak management is heavily reliant on vaccines and antiviral treatments [1]. While the development of novel vaccines and monoclonal neutralizing antibodies is often long, laborious, and unprofitable, polyclonal neutralizing antibodies remain effective disease management option [2].

Chicken egg yolk immunoglobulins (IgYs) are a homolog of mammalian IgGs. IgYs possess therapeutic potential with many advantages compared with IgG [3]. Moreover, IgYs can readily be generated at low cost in large quantities with minimal environmental imact by using egg-laying hens. IgYs showed neutralizing activity in vitro and in vivo experiments against SARS-CoV, influenza virus, Ebola virus, Zika virus, Dengue virus, human norovirus, and SARS-CoV-2, and had favorable safety profiles [4]. IgYs are fast-

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acting. They can neither bind to Fc receptors nor activate complement components in human; therefore, an exacerbation of viral diseases through antibody-dependent enhancement could be potentially avoided.

Here, we report a Norwegian platform for development of IgYs with powerful therapeutic potential. We showed that anti-RBD IgYs efficiently bind RBD of S protein of SARS-CoV-2 in situ, neutralize the virus in vitro, and significantly lower viral replication with minimal effect of immune-related gene expression in vivo. The IgY-based approach could have powerful treatment potential, which can be leveraged for use in response to wild type viruses, their immune-evading or drug-resistant variants filling the time between virus identification and vaccine development with life-saving countermeasures.

2. Materials and Methods

2.1. Preparation and Quantification of Anti-RBD IgY

RBD antigen (His-tagged RBD of SARS-CoV-2/Wuhan Spike glycoprotein, The Native Antigen Company, REC31882) was diluted in PBS to obtain 20 µl volume and mixed and emulsified with 180 µl Imject™ Freunds Complete adjuvant (Thermo Scientific). Each layer (Lohmann LSL Classic) was immunized twice 14 days apart via intramuscular injections (breast muscle) with a total volume of 200 µl. Both immunizations were performed using 10 µg RBD per layer per immunizaton. 14 days after the second immunization eggs were collected. As a negative IgY control, eggs were collected before the layers were immunized (non-immune IgY). IgY was extracted from the yolk using the water dilution method. Yolk was separated from the egg white and added 7x volumes of type 1 quality water. Using 1 N HCl pH was adjusted to 5 before mixing thoroughly. The water diluted yolk was frozen at -20 ^{0}C. The thawed solution was filtered through Whatman Grade 52 Filter Paper for lipid extraction. The clear filtrate was added 8,8% NaCl and using 1 N HCl the pH was adjusted to 4 to precipitate IgY. The solution was stirred gently for 2 h before centrifugation at 3800 x g for 20 min. The pellet, consisting of crude extract of precipitated IgY antibodies, were solubilized in PBS pH 7.

2.2. Indirect ELISA assay

The wells of ELISA plates were coated with the HEK-293 expressed RBD antigens at a concentration of 20 ng/well. Then serial dilutions of IgY's were added to the wells. As a secondary antibody 1:20 000 dilution of HRP-conjugated goat-anti-chicken-IgY (Immuno-Reagents) was used.

2.3. Anti-RBD IgY Testing and Data Quantification

All experiments with viruses were performed in BSL2 or BSL3 laboratories, in compliance with the guidelines of the national authorities, and under appropriate ethical and safety approvals. Standard operational procedures were approved by the institutional safety committee.

The propagation of wild-type SARS-CoV-2 (Slovakia/SK-BMC5/2020) and engineering of recombinant mCherry-expressing SARS-CoV-2 strains (SARS-CoV-2-mCherry) have been described previously [5-10]. To quantitate the production of infectious virions, we titered the viruses using plaque assays [5-9].

Approximately 4 × 10^4 Vero-E6 cells were seeded per well in 96-well plates. The cells were grown for 24 h in DMEM supplemented with 10% FBS and Pen–Strep. The medium was then replaced with DMEM containing 0.2% BSA, Pen–Strep, and the IgY preparation in 3-fold dilutions at 7 different concentrations. No IgY preparation was added to the control wells. The cells were infected with SARS-CoV-2-mCherry strains at an moi of 0.01 or mock. After 48 h of infection, drug efficacy on SARS-CoV-2-mCherry infected cells was measured with fluorescence, as described previously [11]. After 48 h of infection, a CellTiter-Glo (CTG, Promega, Madison, WI, USA) assays were performed to measure cell viability as described previously [11-13].

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The half-maximal effective concentrations (EC_{50}) were calculated using drugvirus.info server [14], based on the analysis of the viability of infected cells by fitting drug dose–response curves using a four-parameter (*4PL*) logistic function *f(x)*:

$$f(x) = A_{min} + \frac{A_{max} - A_{min}}{1 + (\frac{x}{m})^{\lambda}}, \quad (1)$$

where *f(x)* is a response value at dose x, A_{min} and A_{max} are the upper and lower asymptotes (minimal and maximal drug effects), m is the dose that produces the half-maximal effect (EC_{50} or CC_{50}), and λ is the steepness (slope) of the curve. The relative effectiveness of the drug was defined as the selectivity index ($SI = CC_{50}/EC_{50}$).

To quantify each drug response in a single metric, a drug sensitivity score (*DSS*) was calculated as a normalized version of the standard area under dose–response curve (*AUC*), with the baseline noise subtracted, and the normalized maximal response at the highest concentration (often corresponding to off-target toxicity):

$$DSS = \frac{AUC - t(x_{max} - x_{min})}{(100 - t)(x_{max} - x_{min}) \log_{10} Amin'}, \quad (2)$$

where activity threshold t equals 10%, and DSS is in the 0-50 range [15,16].

2.3. Prophylactic Study of anti-RBD IgY Against SARS-CoV-2 Infection in Hamsters

The *in vivo* hamster study was performed at OncoDesign Biotechnology facilities in Villebon-sur-Yvette, France. Eighteen Syrian Golden Hamsters (6–8-week-old males) were randomly distributed to three groups. First group was treated with RBD-IgY (4,6 mg IgY/dose) intranasally 1 h before infection and then twice per day for 3 days (total 9 doses per animal). The Slovakia/SK-BMC5/2020 virus (10^5 pfu $TCID_{50}$ per animal) was introduced intranasally at Day 0. Group 2 was infected with the virus (10^5 pfu $TCID_{50}$ per animal) at Day 0 but IgY was not administered. Group 3 remained uninfected and untreated. Animal viability, behavior and clinical parameters were monitored daily. After 4 days animals were deeply anesthetized using a cocktail of 30 mg/kg (0.6 mL/kg) Zoletil and 10 mg/kg (0.5 mL/kg) Xylazine IP. Cervical dislocation followed by thoracotomy was performed. The superior, middle, post-caval and inferior lobes of lungs were collected and stored in RNAlater tissue storage reagent overnight at 4 °C, and then at -80 °C.

2.4. Gene Expression Analysis

Total RNA was isolated using RNeasy Plus Mini kit (Qiagen, Hilden, Germany) from lungs of Syrian hamsters. RT-PCR was performed using SuperScript™ III One-Step qRT-PCR System kit (commercial kit #1732-020, Life Technologies) with primers ORF1ab_Fw: CCGCAAGGTTCTTCTTCGTAAG, ORF1ab_Rv: TGCTATGTTTAGTGTTCCAGTTTTC, ORF1ab_probe: Hex-AAGGATCAGTGCCAAGCTCGTCGCC-BHQ-1 targeting a region on ORF1ab. Cytokine gene profiling (TNFα, IFNγ, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-21) were performed using qRT-PCR as described [17,18]. RT-qPCR was performed using a Bio-Rad CFX384™and adjoining software. The relative gene expression differences were calculated using b-Actin as control and the results were represented as relative units (RU). Technical triplicates of each sample were performed on the same qPCR plate and non-templates and non-reverse transcriptase samples were analysed as negative controls. Statistical significance ($p < 0.05$) of the quantitation results was evaluated with t-test. Benjamini-Hochberg method was used to adjust the p-values.

3. Results

We immunized layers twice with 14-day interval using 10 µg HEK-expressed RBD of SARS-CoV-2 (Wuhan) or mock per layer per immunization. Eggs were harvested 14 days after the second immunization and IgY was extracted from yolks (Figure 1a). IgY response was measured by indirect ELISA assay (Figure 1b). IgY bound to RBD with EC50

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value of 1.9 ug/mL. IgY from non-immunized layers did not bind RBD. These results demonstrate that our immunization protocol led to production of RBD-specific IgYs.

We next examined whether anti-RBD IgY can inhibit SARS-CoV-2 infection and protect cells from virus-mediated death. We tested the antiviral efficacy and toxicity of anti-RBD and control IgYs by monitoring SARS-CoV-2-mediated mCherry expression and viability of Vero-E6 cells as described previously [4]. We observed that anti-RBD IgY but not control non-immune IgY reduced SARS-CoV-2-mediated mCherry expression and rescued cells from virus-mediated death (Figure 1c). According to both assays the EC50 values were in 0.1-0.3 mg/ml range and SI were >10, indicating to high selectivity of anti-RBD IgY. Importantly, IgYs raised against RBDs of S of Wuhan and Omicron variants were also able to neutralize Omicron virus (Fig. S1).

Next, we examined whether anti-RBD IgY can affect the replication of SARS-CoV-2 in vivo. Eighteen 6-8-week-old male Syrian Golden Hamsters were randomly distributed to three groups. First group was inoculated with SARS-CoV-2 and treated intranasally with anti-RBD IgY 1 h before inoculation and then twice per day for 3 days. After 4 days animals were deeply anesthetized, the cervical was dislocated and thoracotomy was performed. The lungs were collected, and RNA was extracted from superior lobes of right lungs.

We used RT-qPCR to analyze expression levels of viral ORF1ab gene in lungs of Syrian Golden Hamsters at Day 4 after infection with SARS-CoV-2. The values were normalized to b-Actin gene expression. We found that anti-RBD IgY significantly reduced relative levels of ORF1ab in lungs of infected animals. We also profiled expression of several immune-related genes in lungs of three groups of animals. We observed significant ($p<0.05$) change in relative IL-2 gene expression and substantial ($p<0.1$) changes in IL-10 and IL-12 levels between treated and non-treated virus-infected animals. This suggests that treatment with anti-RBD IgY slightly imbalances virus-mediated expression of several immune-related genes in the lungs of Syrian Golden hamsters.

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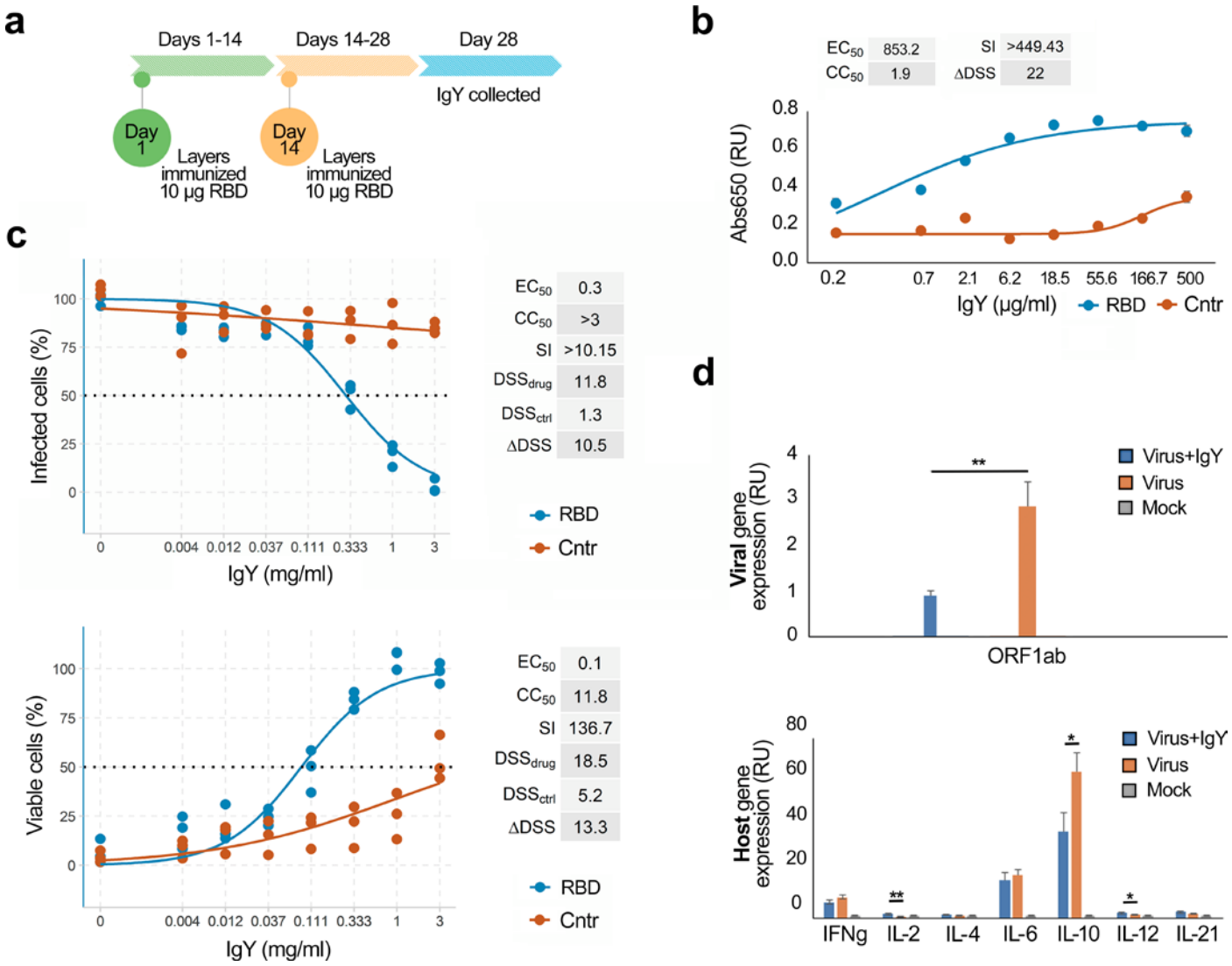

Figure 1. Pilot experiments showing that anti-RBD IgY antibodies produced in eggs neutralize SARS-CoV-2 virus in vitro and attenuate viral RNA synthesis in vivo. (**a**) Layers were immunized twice with 14-day interval using purified RBD of SARS-CoV-2. IgYs were extracted from eggs harvested 14 days after the second immunization. (**b**) Binding of anti-RBD or control non-immune IgY to RBD was measured by indirect ELISA. Mean; n = 2. (**c**) Neutralization capacity of anti-RBD or control non-immune IgY was measured by monitoring mCherry fluorescence or viability of Vero-E6 cells after 48 h of infection with mCherry-expressing SARS-CoV-2 strain. Mean ± SD; n = 3. (**d**) Effect of anti-RBD IgY on the expression of SARS-CoV-2 and host cytokine genes in length of Serian Golden hamsters. Mean ± SD; n = 6. * - $p<0.1$, ** - $p<0.05$ (Wilcoxon test).

4. Discussion

Currently, neutralizing antibodies are still largely eschewed for the treatment of emerging and re-emerging viral infections in favor of vaccines and antiviral drugs, due to the time and cost associated with their development. This is because many natural sources for production of neutralizing antibody have not been fully explored or understood. Chicken polyclonal egg yolk IgYs are easy to produce, and low cost.

Here, we report Norwegian platform for antiviral IgY development. We used the platform to demonstrate low toxicity and neutralization activity of IgYs raised against RBD of S protein of SARS-CoV-2 in vitro and in vivo. At the same time the treatment allowed activation of several innate immune-related genes, that mediate the development of adaptive immune responses.

Further studies will be required to determine if the IgY-based approach that was witnessed for in situ, in vitro and in vivo models could be translated in clinical trials. If successful, IgY-based antiviral therapeutics could be valuable due to their lowered potential for adverse side effects (except for people with egg-allergies), and thus be useful in treating patients with emerging and re-emerging viral diseases. In addition, the IgY could be delivered through inhalation and other routes, leading to greater ease of treatment. Furthermore, IgY -based technology could be used in future viral outbreaks in Norway, given the demonstrated proof-of-concept.

Thus, our proof-of-concept pilot study shows that research on potential antiviral IgYs can have significant local as well as global impact, by increasing protection of the population against emerging and re-emerging viral diseases and filling the time between virus identification and vaccine development with life-saving countermeasures.

5. Conclusions

Our pilot study demonstrated anti-SARS-CoV-2 activity of chicken IgYs. It illustrates an important approach to the development of fast, inexpensive, and effective treatments against SARS-CoV-2. We believe further development of antiviral IgY technology could lead to practical therapeutic options against many viruses.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Figure S1. IgY antibodies raised against receptor-binding domains (RBD) of spike glycoproteins of Wuhan and Omicron variants neutralize SARS-CoV-2/Omicron virus in vitro.

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Data Availability Statement: All data generated or analyzed during this study are included in this published article and its supplementary information files (Figures S1).

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Conflicts of Interest: The authors declare a conflicts of interest. Lasse Evensen is affiliated with Norimun AS, whereas Gorm Sanson is affiliated with Felleskjøpet Fôrutvikling AS. These authors declare financial interests.

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