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Electronic Supplementary Material

Anti-SARS-CoV-2 IgY Isolated from Egg Yolks of Hens Immunized with Inactivated SARS-CoV-2 for Immunoprophylaxis of COVID-19

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Materials and Methods

Cell, Virus, and Reagents

SARS-CoV-2 (20SF014-SARS-CoV-2) was stored at −80 °C at the Laboratory of Guangdong Provincial Center for Disease Control and Prevention (Guangzhou, China). Vero-E6 cells were cultured in modified Eagle medium (MEM; Gibco/Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Corning Inc., Corning, NY, USA), 100 U/mL penicillin (Sigma-Aldrich/Merck KGaA, Darmstadt, Germany), and 100 μg/mL streptomycin (Sigma-Aldrich/Merck KGaA). Vero-E6 cells were cultured in virus-containing medium at 37 °C in a 5% CO_2-humidified incubator. When Vero-E6 cells containing the viruses reached ~80% confluence CPE, cells were frozen-thawed at −80 °C, centrifuged at 8,000 ×*g* for 10 min at 4 °C, and filtered through a 0.22-μm filter for storage. Then, formaldehyde-inactivated SARS-CoV-2 was used to immunize the hens. HRP-labeled rabbit IgG against chicken IgY was purchased from the Earth OX Company.

$TCID_{50}$ Assay

Virus strains were serially diluted (from 10^{-1} to 10^{-10}) with DMEM supplemented with 10% FBS and titrated at 100 μL/well on RD monolayer cells that had been seeded in a 96-well plate at a density of 1×10^4 cells/well. The

virus-infected cells were incubated for 48 h at 37 °C in a 5% CO_2-humidified incubator before they were observed for any cytopathic effects under a microscope (IX73; Olympus Corporation, Tokyo, Japan). The $TCID_{50}$ value of the virus strains was determined according to the Reed-Muench formula.

Immunization of Hens

A total of 300 Lohmann pink-laying healthy young (20 weeks old) hens were purchased from Sichuan Zhengda Egg Industry Co. Ltd. (Meishan, China). Three or four chickens were raised in each cage and had access to food and water. The laying chickens were divided into the immunization group and the control group. After the SARS-CoV-2 antigen was mixed with an equal amount of Freund's complete adjuvant and fully emulsified, chickens from the immunization group were subcutaneously administered the antigen solution on both wings (0.5 mL/hen). In the control group, chickens were administered a mixture of saline and Freund's complete adjuvant (0.5 mL/hen). After 2 weeks, one booster immunization was performed, and a second booster immunization was done 3 weeks later. One week after the final immunization, the eggs were used to collect for extraction of crude IgY antibodies.

Isolation and Purification of IgY

Fresh eggs were cleaned with purified water and 75% alcohol, and the yolks were separated and collected. The egg yolk was diluted with purified water (1:9) and mixed, adjusted to pH 5.0 and stored overnight at 4 °C. The mixed solution was centrifuged at 8,000 rpm for 40 min at 4 °C, the ammonium sulfate was added to supernatant to achieve a final saturation of 19%. Following this, the solution was incubated overnight at 4 °C. After centrifugation at 4,000 $\times g$ for 10 min, the supernatant was discarded and the protein precipitate was dissolved in the 0.01 mol/L citrite-phosphate buffer. Subsequently, the solution was pasteurized at 60 °C for 10 hours. The IgY purified from eggs of the immunization group was the specific IgY (anti-SARS-CoV-2 IgY, which marked that Lot No. B-S2202005006), and that from control group was the isotype control IgY. The purified IgY was subjected to ultrafiltration using an average molecular weight of 100 kDa ultrafiltration system (Pall, USA) to desalt and concentrate the antibody. The purified IgY was used in subsequent experiment.

SDS-PAGE and Western Blotting

The purified IgY samples were quantified by BCA assay (PierceTM BCA Protein Assay Kit. Cat. no. 23227). Samples of IgY and 5× loading buffer (4:1) were heated in a metal water bath at 95 °C for 10 min and placed on ice for an additional 10 min. Samples (10 μL/lane) were separated via SDS-PAGE on a 12% gel at a constant current of 45 mA for 50 min. Following protein separation, the separated proteins were transferred onto polyvinylidene fluoride membranes and blocked with 5% skimmed milk at room temperature for 90 min. The membranes were washed five times with Tris-buffered saline containing 0.05% Tween-20 (TBST) and then incubated with HRP-labeled rabbit anti-chicken IgY antibody (1:10000) overnight on a shaking incubator at 4 °C. The membranes were washed five times with TBST, and protein bands were visualized using an enhanced chemiluminescence western blotting kit (Thermo Fisher Scientific Inc.).

ELISA to Measure the Binding of IgY to the RBD or S1 Protein

We performed homemade ELISA by coating 96-well microtiter ELISA plates with anti-SARS-CoV-2-IgY (10

μg/mL) or the isotype control (10 μg/mL) for 2 h at 37 °C. After the plates were washed two times with PBST (5 min/time), 5% BSA was used to block the reaction at 37 °C for 2 h. After blocking, the plates were washed two times (for 5 min each time). Four-fold diluted Fc-tag S1 (Kactus Biosystemscompany, cat. no.COV-VM5S1) or RBD (Kactus Biosystemscompany, cat. no.COV-VM5BD) subunit was added in the specific well (the initial concentration of S1 and RBD was 5 μg/mL and 200 ng/mL, respectively) and incubated at 37 °C for 1 h. The plates were washed three times (5 min each time), and HRP-conjugated IgG (1:5000; cat.GR197135-3, Abcam) was added to the wells and incubated at 37 °C for 1 h. After 1 h, the plates were washed four times (5 min each time), and 50 μL TMB was added. Finally, the reaction was blocked after 5 ± 2 min by addition of 1 mol/L sulfuric acid at a concentration of 50 μL/well (Carlo Erba Reagents Srl, Cornaredo, Milan, Italy). The absorbance of each sample was measured at 450 nm with a microplate spectrophotometer (Multiskan EX; Thermo Fisher Scientific, Waltham, MA, USA).

Competitive ELISA

For competitive ELISA, the ELISA microplates were coated with His-tagged ACE2 at a concentration of 2 μg/mL with a volume of 50 μL per well and incubated at 37 °C for 2 h in ELISA coating buffer (carbonate bicarbonate buffer, pH 9.2 ± 0.2). After the plates were washed two times with PBST (5 min each time), 5% BSA was used to block the reaction at 37 °C for 2 h. After blocking of the reaction, the plates were washed two times (5 min each time). Then, the different concentrations of anti-SARS-CoV-2-IgY or isotype IgY were reacted with Fc-tagged-S1 (1 μg/mL) or Fc-tagged-RBD (100 ng/mL) at 37 °C for 1 h. Finally, the liquid mixture of IgY/RBD or IgY/S1 was added in the 96-well microtiter ELISA plates and incubated at 37 °C for 1 h. The plates were then washed three times (5 min each time), and 50 μL/well of rabbit anti-human HRP-conjugated IgG was added (1:3000; cat.GR197135-3, Abcam) to the wells and incubated at 37 °C for 1 h. Then, the plates were washed four times (5 min each time), and 50 μL TMB was added in each well. Finally, the reaction was blocked after 5 ± 2 min by the addition of 1M sulfuric acid at a concentration of 50 μL/well (Carlo Erba Reagents Srl, Cornaredo, Milan, Italy). The absorbance of each sample was measured at 450 nm with a microplate spectrophotometer (Multiskan EX; Thermo Fisher Scientific, Waltham, MA, USA).

Cell-cell Fusion Assay

The cell-cell fusion assay was performed as previously described. In brief, HuH-7 cells were used as target cells, while 293T cells transfected with the S protein expression vector pAAV-SARS-CoV-2-S/GFP were prepared as effector cells. Effector cells were added to target cells and co-cultured in DMEM containing 10% FBS, without trypsin, for 2 h. After incubation, five fields were randomly selected in each well to count the number of fused and unfused cells under an inverted fluorescence microscope (Nikon Eclipse Ti-S).

Inhibition of Pseudotyped SARS-CoV-2

293T cells were co-transfected with pNL4-3.luc.RE (the luciferase reporter-expressing HIV-1 backbone) and pcDNA3.1-SARS-CoV-2-S (encoding for the SARS-CoV-2 S protein) using VigoFect (Vigorous Biotechnology, Beijing, China). Pseudotyped particles were efficiently released in the supernatant. The supernatant was harvested at post-transfection 72 h, centrifuged at 3,000 ×*g* for 10 min, and stored at −80 °C. To detect the inhibitory activity of anti-SARS-CoV-2-IgY antibody against SARS-CoV-2 PsV infection, HuH-7 cells were plated at a density of

10^4 cells per well in a 96-well plate one day prior to infection. SARS-CoV-2 PsV was mixed with an equal volume of anti-SARS-CoV-2-IgY serially diluted with PBS at 37 °C for 30 min and the mixture was transferred to the HuH-7 cells. The medium was changed after 12 h and incubation was continued for 48 h. Luciferase activity was analyzed with the Luciferase Assay System (Promega, Madison, WI, USA).

Inhibition of Live SARS-CoV-2 Replication

The inhibition assay for live SARS-CoV-2 was performed in a biosafety level 3 (BSL3) facility at the Laboratory of Guangdong Provincial Center for Disease Control and Prevention (Guangzhou, China). Anti-SARS-CoV-2-IgY at different concentrations was mixed with SARS-CoV-2 (100 $TCID_{50}$) for 2 h. At the same time, the isotype IgY was used as a control for this experiment. Subsequently, Vero-E6 cells were inoculated with 100 μL of the mixture and incubated at 37 °C in a 5% CO_2-humidified incubator for 3–5 days to promote antibody binding to the viruses. Following this, Cell Counting Kit-8 (CCK8; Dojindo, Kumamoto, Kyushu, Japan) assay was used to examine the cytopathic effect.

***In vivo* Imaging**

The anti-SARS-CoV-2-IgY solution was labeled with fluorescent molecules (Cy7-SE; Fanbo Biochemicals Company, Beijing, China), which were administered to 4- to 8-week-old Balb/c female mice via oral spray and nasal drip. Mouse of the two groups were fed a normal diet as per the standard protocol. In the oral spray group, each mouse was immunized with 0.11 mL of antibody solution, and in the nasal drip group, each mouse was immunized with 10 μL of the solution in each nasal cavity. After administration of the antibody solution at 1 h, 2 h, 4 h, 6 h, 8 h, 12 h, and 24 h, images were captured with the IVIS Imaging System (Xenogen, Alameda, CA, USA) for 10–120 s (bin size = 2). Data were normalized to bioluminescence levels at the initiation of treatment for each mouse.