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Passive protection effect of chicken egg yolk immunoglobulins on enterovirus 71 infected mice

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A B S T R A C T

The objective of this study is to evaluate the passive protective efficiency of immunoglobulin in yolk (IgY) specific against human enterovirus type 71 (EV71). The antibody was raised by intramuscular immunization to 10 White Leghorn hens, with inactivated human EV71 serving as the antigen. The titer and specificity of the antibody were analyzed from purified IgY in the egg yolks of immunized hens. Results indicate that the titer of IgY specific against EV71 increased from the third week after the first immunization. The content of total IgY was 190 ± 26 mg/yolk, with an average concentration of specific IgY of 6.34 ± 3.38 mg/yolk in the eggs from 3 to 18 wk after immunization. The results of the neutralization effect of specific IgY in EV71-challenged mice demonstrate that the EV71-specific IgY, either by intraperitoneal injection or oral administration, was able to significantly reduce the morbidity and mortality in EV71 infected mice pups.

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1. Introduction

Researchers have extensively studied passive immunization in both humans and animals using specific antibodies to protect against pathogens. An increase in antibiotic-resistant bacteria and the desire to treat pathogens that do not respond to antibiotics, such as viral pathogens, has prompted many researchers to use antibodies as alternatives to antibiotics [1]. IgY (immunoglobulin in yolk) antibodies are the predominant serum immunoglobulin in birds, reptiles, and amphibians, and are transferred from serum to egg yolk in the females to confer passive immunity to their embryos and neonates [2]. The potential of orally administered IgY for the prevention and treatment of many pathogens has been studied for many years, for Escherichia coli [3], Helicobacter pylori [4], Salmonella [5], Rotavirus [6], Staphylococcus [7], Streptococcus mutans [8], Yersinia [9], Coronavirus [10], and the Porcine epidemic diarrhea virus [11]. This paper evaluates the efficiency of IgY against enterovirus 71 (EV71).

Enterovirus 71 belongs to the human enterovirus A family of Picornaviridae. These virions consist of a non-enveloped capsid surrounding a core of single-stranded, positive-polarity RNA approximately 7.5 kb in size [12]. Since the initial description of EV71 in 1974 [13], outbreaks of this virus have been identifi
ced periodically in countries throughout the world, including the USA, Australia, Sweden, Japan, Bulgaria, Hungary, Hong Kong, and Malaysia [14]. In 1998, an outbreak of EV71 infections occurred in Taiwan, in which 405 children were hospitalized and 78 died [15]. EV71 infections are generally mild, like hand-foot-and-mouth disease (HFMD) and herpangina, and even fatal encephalitis in neonates [16–18]. In recent years, some efforts have been made to control EV71 infections. The most promising antiviral agents for EV71 are WIN-group compounds, which have undergone clinical trials [19,20]. In addition, a research group from Taiwan has developed two candidate EV71 vaccines, including a formalin-inactivated whole virus vaccine and a VP1 expressing DNA vaccine [21]. The efficacy of both vaccine constructs is currently being tested in animal models.

Several approaches have been attempted in the treatment of viral diseases. Researchers tried to block the replication of virus through binding the capsid of virus with some chemicals, such as pyridazinamines [22], phenoxy imidazoles [23], or pleconaril [24,25]. However, the treatment of EV71 with pleconaril was not significant, the binding of pleconaril with capsid was limited under higher viral concentration in vitro [26]. A specific antibody which could bind with the virus and hence reduce the contact of virus with host cells is an alternative choice. Human’s immunoglobulin has been applied in the treatment of EV71 infections in babies whose immune system are not well-developed [27,28]. There is no routine therapy for the treatment of EV71 infection with human’s
immunoglobulin so far. However, some animal model of EV71 challenged mouse has been developed, and provided some information about the protective effects of the neutralizing antibody on EV71 infection [29]. Compared to traditional antibody production, chicken as bio-factory can produce higher yield of IgY antibodies than mammals’ IgG. A chicken can lay 280 eggs in a year and an egg yolk contains 100–200 mg of IgY antibodies. This study was subjected to produce IgY against enterovirus 71 (anti-EV71 IgY) and evaluated the inhibition effects of specific IgY on EV71, including in vitro virus neutralization test and in vivo ICR mice model. This study also provided an animal model for the application of IgY in the cure or prevention of EV71.

2. Materials and methods

2.1. Virus strains and cells

The EV71 strains 4643 and MP4. Strain 4643 was originally derived from a patient with EV71 encephalitis [30], while strain MP4 was a mouse-adapted enterovirus 71 with increased virulence in mice, and it was generated after four serial passages of the 4643 strain [31]. EV71 stock virus of strains 4643 and MP4 were grown in RD (rhabdomyosarcoma) cells, which were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, U.S.A.), containing 10% fetal bovine serum (FBS, Invitrogen, U.S.A.), 2 mM of L-glutamine (Invitrogen, U.S.A.), 100 IU/ml of penicillin (Invitrogen, U.S.A.), and 100 μg/ml of streptomycin (Invitrogen, U.S.A.).

2.2. Immunization of chickens

All animals received humane care as outlined in the guide for the care and use of experimental animals and viral challenge (Institutional Animal Care and Use Committee; IACUC Approval No. 9400021). Four 6-month-old White Leghorn laying hens, obtained from Livestock Research Institute, Council of Agriculture, Executive Yuan, Taiwan, were utilized for the production of anti-EV71 IgY. A formalin-inactivated EV71 of strain 4643 was utilized as the antigen. One milliliter of EV71 antigen (400 μg/ml, 5.4 × 10^6 pfu) was emulsified with an equal volume of Freund complete adjuvant and immunized intramuscularly to chickens at 4 sites in the breast muscle. Four booster injections with Freund’s incomplete adjuvant were given at weeks 2, 4, 6, and 13 after the first immunization. The eggs were collected daily from the first day to 3 weeks after the last immunization, and stored at 4 °C. The egg yolk was separated, pooled, and kept at −20 °C prior to IgY purification, and all egg yolks from each chicken for each week were pooled into one analysis sample.

2.3. Isolation and purification of IgY

The isolation of IgY was carried out as described by Akita and Nakai [32,33], with some modifications. The egg yolk was first mixed with one of nine volumes of cold distilled water (acidified with 0.1 N HCl to pH 5.0) and stored overnight at 4 °C. The mixture was then centrifuged at 3125 × g at 4 °C for 40 min to obtain the water soluble fraction (WSF). The WSF was collected and filtered to remove solids. The resulting IgY-containing WSF was further purified by ultra filtration using Amicon Ultra-15 filter (PL-100, Millipore), condensing the sample to 1/30 to 1/40 of its original volume. These WSF concentrates were then subjected to the virus neutralization test and mice challenge experiments.

2.4. ELISA (enzyme-linked immunosorbent assay)

The antibody activity of anti-EV71 IgY was determined using the ELISA method described by Lee et al. [34], with some modifications. Briefly, microtiter plates were coated with 100 μl of inactivated EV71 4643 antigen (10 μg/well), while control wells were coated with rabbit anti-chicken IgY antibody (10 μg/ml, Sigma C2288). The plate was then incubated overnight at 4 °C. After washing with PBS–TWEEN 20 buffer, 2% BSA blocking was conducted overnight at 4 °C. The wells were then washed with PBS–TWEEN 20 buffer. Next, 100-fold diluted WSF was added to the sample wells (100 μl/well) for testing. WSF from the same chicken before immunization was used as a control. To generate the standard curve, wells were filled with 100 μl serial-diluted pure chicken IgY at a concentration from 0.015 μg/ml to 1 μg/ml (Promega, G116A) and incubated at 4 °C for overnight. After washing with PBS–TWEEN 20 buffer, 100 μl of alkaline phosphate-conjugated goat anti-chicken IgY (Promega, G115A) was added to the wells and incubated at 37 °C for 2 h. After washing with PBS–TWEEN 20 buffer, 100 μl of disodium p-nitrophenyl phosphate was added to each well as a substrate (Sigma, N9389) and allowed to react at 37 °C for 10 min. The absorbance was then measured at 405 nm using a microplate reader (Multiskan MS; Thermo Labsystems). The resulting absorbance of standard curves provided a relative measurement of anti-EV71 IgY concentration. For the total IgY determination, each well on the microtiter plate was first coated with 100 μl of rabbit anti-chicken IgY antibody (10 μg/ml, Sigma C2288), to which 100 μl of 10,000-fold diluted WSF was then added. The following experiments were performed following the same protocol described above.

2.5. Western blot analysis

The EV71 was run on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrically onto a nitrocellulose membrane. The membrane was divided into 3 parts and soaked in blocking buffer (5% nonfat dry milk in TBST) for 30 min at room temperature, then incubated with anti-EV71 or non-specific IgY for 1 h. After washing 4 times with TBS-T, the membranes were incubated with peroxidase-conjugate goat anti-chicken IgY (1000-fold in blocking buffer for each) at room temperature for 1 h. Immunoreactivity was detected by incubating the membranes with 0.02% (W/V) 4-methoxy-1-naphthol in TBS and 0.02% (V/V) H2O2.

2.6. TCID50 and EV71 neutralization tests

The TCID50 of MP4 virus and the neutralization titer of anti-EV71 IgY were determined using the method described by Yu et al. [35]. In the TCID50 tests, each well–well 8 × 10^3 RD cell was added to a microplate well, followed by the 10-fold serial diluted virus. The 50% tissue culture infective dose was determined after incubation under 5% CO2 at 37 °C for 7 days. In the neutralization tests, each 50 μl sample of serial diluted anti-EV71 IgY concentrate solution was mixed with 50 μl of 100 TCID50 EV71 in a microplate well. Two hours later, the RD cell suspensions (8 × 10^3 cell/well) were added and further incubated under 37 °C and 5% CO2 for 7 days. Neutralization antibody titers were then identified as the highest dilution of purified IgY solution that completely inhibited virus growth.

2.7. EV71 infection and IgY protection tests

The challenge test of EV71 of the mouse model in this study was modified from Wang et al. [30]. Trials 1–4 were designed to test the effect of IgY with different neutralization titers on mortality in suckling mice. Each one-day-old ICR strain mouse was intraperitoneally (i.p.) inoculated with 1 × 10^6 pfu of MP4 virus, which is a mouse-adapted 4643 strain of EV71. Thereafter, IgY with different neutralization titers (64, 128, 256, and 512) was injected i.p. into the challenged mice 1–3 days after inoculation (dpi). The dosage of IgY was determined to be 1 μg/mouse.
Table 1
Purity of IgY after different procedure of purification.

| Purification step          | Total protein (mg/egg) | IgY (mg/egg) | Purity (%) |
|----------------------------|------------------------|--------------|------------|
| Water soluble solution (WSF) | 595 ± 37a              | 190 ± 26a    | 32 ± 6     |
| Ultrafiltration            | 231 ± 25a              | 151 ± 21a    | 65 ± 5     |

* The value was derived from the concentration of the water soluble fraction (WSF), based on an average WSF of 150 ml per egg from all chicken eggs between 3 and 18 weeks, was mean as average ± SD.

Fig. 1. The content of EV71 specific IgY per egg yolk during the immunization period. Values are shown as average data from eggs in the same week for all immunized chickens. Vertical bars indicate the standard deviation.

was 100 μl per mouse. In trials 5 and 6, the IgY treatment dates were 2–4 dpi and 4–6 dpi, respectively. Trials 7–8 were used to evaluate the protection effect of anti-EV71 IgY by oral treatment. Specifically, trials 7 and 8 used a 24-gauge feeding tube to orally inoculate mice of 4–5 d (2.5–3.0 g) and 5–6 d old (3.0–3.5 g), respectively. The mice were given 100 μl of MP4 virus (3 × 10^6 pfu/mouse) after 12-h of fasting. For each pup, 100 μl of anti-EV71 IgY with a titer of 512 was orally administered either 1 h before or 1 h after viral challenge. All mice were checked daily for their body weight and the syndromes of EV71 infection, including limb paralysis and abnormal hair coat until 2 weeks after viral infection. Morbidity and mortality data was collected for two weeks after the viral challenge.

3. Results

3.1. Production of IgY against EV71

The concentration of total IgY and specific anti-EV71 IgY was analyzed by ELISA. The content of IgY in the WSF of egg yolk was investigated for a period of 18 weeks, from the first week of immunization until 6 weeks after the last booster injection. The average content of total IgY in each yolk was 190 ± 26 mg (Table 1), which was 32% of the egg yolk’s total protein. After condensation by ultrafiltration, the total protein per egg was 231 ± 25 mg on average, and the purity of total IgY increased to 65%.

The content of specific anti-EV71 IgY increased gradually from the third week after the first immunization, as Fig. 1 indicates. A variation in immune response among individual hens was noticed from the large standard deviation. The average anti-EV71 IgY concentration reached 6.34 ± 3.38 mg per yolk between the third to eighteenth weeks, equivalent to 3.33% of the total IgY.

3.2. The neutralization titer determination of specific IgY

The neutralization titer of specific anti-EV71 IgY was determined by the cytopathic effect (CPE) of EV71 on RD cells (Fig. 2). The resulting neutralization titers of specific IgY obtained from eggs of all immunized chickens ranged from 0 to 2048 (data not shown). Comparing the neutralization titers to the contents of specific IgY in the respective yolks showed no significant cor-

Table 2
Neutralization titers of EV71 specific IgY from different chickens.

| Chickens | Concentration of specific IgY against EV71a (μg/ml) | Neutralization titerb |
|----------|-----------------------------------------------------|-----------------------|
| YA1 (8W) | 9.6                                                 | 0                     |
| YA2 (17W)| 33.4                                                | 16                    |
| YA3 (15W)| 37.8                                                | 0                     |
| YA4 (8W) | 45.5                                                | 0                     |
| YB1 (6W) | 40.1                                                | 0                     |
| YB2 (8W) | 1.7                                                 | 64                    |
| YB3 (9W) | 50.2                                                | 512                   |
| YC1 (15W)| 28.9                                                | 64                    |
| YC2 (15W)| 19.9                                                | 256                   |
| YC3 (8W) | 16.8                                                | 0                     |
| YC4 (8W) | 20.7                                                | 16                    |
| YE1 (16W)| 49.1                                                | 2048                  |
| YE2 (11W)| 46.5                                                | 256                   |
| YE3 (15W)| 123                                                 | 16                    |
| YE4 (7W) | 66.5                                                | 128                   |

* The concentration of WSF specific IgY against EV71 was determined using the ELISA method.

b The IgY-containing WSF was further purified by ultra filtration using Amicon Ultra-15 filter (PL-100, Millipore), condensing the sample to 1/40 of its original volume. These WSF concentrates were then subjected to the virus neutralization test.
relation between these two sets of data \( r = -0.396 \), data not shown).

Western blotting analysis of the immunoreactivity of anti-EV71 IgY. The EV71 was run SDS-PAGE and transferred onto nitrocellulose filter and probe with anti-EV71 IgY and non-immunized IgY. The EV71 was detected of ∼52 kDa molecular mass (Fig. 3A) and anti-EV71 IgY was specifically bound to the proteins with molecular weight corresponding of EV71 virus (Fig. 3B).

3.3. Neutralization effect of specific IgY in EV71 challenged mice

In the first part of the animal tests, ICR mice showed both limb paralysis and abnormal hair growth after EV71 challenge in the IP model (Fig. 4). In trials 1–6 was conducted to investigate the effect of IP injected specific IgY on curing IP EV71-challenged mice (Table 3). When one-day-old ICR mice were challenged with EV71 of strain MP4 with a dose of $10^5$ pfu per mouse, the average morbidity was $96.5 \pm 6.1\%$ in the control group. The resulting mortality was $88.0 \pm 8.4\%$ based on the challenged mice, or $91.2 \pm 7.6\%$ when based on illness in mice injected with only non-immunized IgY (Table 3). We IP administrated the purified specific IgY derived from condensation of the immunized yolk WSF to EV71-challenged mice. This administrated was applied continuously for 3 days, from the day after viral infection (1 dpi) to the third day post infection (3 dpi). The therapeutic effects of IgY varied according to the neutralization titers of the specific IgY administered. The infection rate was 100% when the titer of specific IgY was 64, which is same infection rate as in the control group (Table 3). The treatment of IgY with a titer of 64 did not significantly reduce mortality in trial 1. However, when the IgY titers were 128 or higher, the morbidities reduced to 20%, 7%, and 0%, respectively for IgY treated groups with titers of 128, 256 or 512. Only the group of titer 128 (trial 2) had some deaths after viral infection and IgY injections, with a mortality rate of 10%. Without the treatment of specific IgY, the challenged mice showed high morbidity and mortality. In trials 5 and 6, if the treatment...
Effects of IP challenge on EV71a and IgYb treatment.

Table 3

| Trial no. | Mice no. | EV71 specific IgY titer | IgY IP date (dpi) | Morbidity (%) | Mortality (%) | Mortality of ill mice (%) |
|-----------|----------|-------------------------|------------------|---------------|---------------|--------------------------|
| Controlb | 14       | 64                      | 1–3              | 100 (14/14)   | 92 (13/14)    | 92 (13/14)               |
| 2         | 10       | 128                     | 1–3              | 100 (13/13)   | 77 (10/13)    | 77 (10/13)               |
| Controlb | 3        | 30                      | 1–3              | 100 (12/12)   | 100 (12/12)   | 100 (12/12)              |
| 4         | 21       | 512                     | 1–3              | 7 (2/30)      | 0 (0/30)      | 0 (0/2)                  |
| Controlb | 5        | 14                      | 1–3              | 100 (20/20)   | 92 (24/26)    | 92 (24/26)               |
| 6         | 19       | 512                     | 3–5              | 100 (23/23)   | 91 (21/23)    | 91 (21/23)               |
| Trial (Ave ± Std) | 25     | 512                     | 1–3              | 43 ± 42.5%    | 31 ± 37.9%    | 34 ± 32.4%               |
| Controlc  | 6        | 512                     | 3–5              | 94 (17/19)    | 63 (12/19)    | 71 (12/17)               |
| 7–1       | 5        | 16                      | 1–3              | 43 (6/14)*    | 21 (3/14)*    | 30 (3/6)                 |
| 2–4       | 14       | 512                     | 1–3              | 94 (15/16)    | 88 (14/16)    | 93 (14/15)               |
| Controlc  | 20       | 256                     | 1–3              | 100 (26/26)   | 92 (24/26)    | 92 (24/26)               |
| 3–5       | 89 (17/19) | 256                     | 1–3              | 0 (0/21)*     | 0 (0/21)*      | 0 (0/21)                 |
| Controlc  | 16       | 256                     | 1–3              | 100 (23/23)   | 91 (21/23)    | 91 (21/23)               |
| 2–4       | 43 (6/14)* | 512                     | 1–3              | 85 (17/20)    | 80 (16/20)    | 94 (16/17)               |
| Trial (Ave ± Std) | 20     | 512                     | 3–5              | 43.1 ± 42.5%  | 31 ± 37.9%    | 34 ± 32.4%               |
| Control (Ave ± Std) | 19   | 512                     | 3–5              | 96.5 ± 6.1%   | 88.0 ± 8.4%   | 91.2 ± 7.6               |

a IP injection dose: 10^5 pfu/mouse, one-day-old mouse.
b IP injection dose: 100 μl/mouse/day (IgY titer between 64 and 512).
c Non-immunized IgY.
d Differences in proportions morbidity and mortality were tested with the use of the X² statistic (P<0.05).

4. Discussion

In recent years, pathogen-specific IgY has also been demonstrated to be effective in the passive protection of human’s diseases, such as Staphylococcus for holotoxin [7], Rotavirus for diarrhea [36], dental caries caused by S. mutans [37], and H. pylori for gastric ulcers [4].

In this study, we tried to produce the specific IgY against human EV71. This enterovirus was first reported in the United States in 1974 [13] and has subsequently been reported worldwide [38,39]. In Taiwan, some severe cases of human EV71 are reported every year after the devastating EV71 outbreak of 1998 [15]. The seriousness of EV71 infection lies in its complications, which include hand-foot-and-mouth disease, herpangina, aseptic meningitis, poliomyelitis-like paralysis, and fatal encephalitis [40]. Most fatal cases occur in children under 3 years old, and high mortality is correlated with brainstem encephalitis [41].

The specific IgY against EV71 in the egg yolk increased after the first immunization, though there were obvious differences among individual hens. This variation in antibody pro-
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more accessible to the gastric juice and easily exposed to digestive enzymes. The activity of might be influenced by the change of structure and loss of bioactivity [57]. Based on the limitations of oral administration in suckling pups, a higher titer of IgY or a continuous treatment of IgY after viral challenge might achieve greater protection. This possibility is worthy of further research. The results of this study, however, indicate the potential of IgY in the prevention of EV71 infection in young children.

Several studies have been conducted to evaluate the stability of these antibodies. The IgY is not degraded during pasteurization at 60 °C [52] IgY was stable after 30 min in 60–65 °C, but was no longer active after 20 min in 80 °C [53]. So IgY technology being new potential market applications in medicine, public health, veterinary medicine and food safety. A broader use of IgY technology could be applied as biological or diagnostic tool, nutraceutical or functional food development, oral-supplementation for prophylaxis, and as pathogen-specific antimicrobial agents for infectious disease control.

In conclusion, the ICR mice IP challenged with mouse-adapted strain MP4 of EV71 at a dose of 10^6 pfu per mouse induced an EV71 infection resulting in a high mortality rate. However, a survival rate of 98.3% (60/61) was achieved if the challenged mice were IP injected, 1–3 dpi for 3 consecutive days, with a purified IgY antibody with a neutralization titer of 128 or more. When the challenge was carried out orally with a dose of 3 × 10^6 pfu per mouse, the lower mortality of IgY-pretreated group provided the protection could be achieved by the neutralization of virus in the gastrointestinal duct, thereby reducing the mortality of challenged mice.

This is the first study to evaluate the effect of IgY treatment on EV71 infection, and our positive results indicate that further application in the prevention of EV71 is possible. In the form of an egg-yolk-added drink, yolk powder tablet, or capsule, it can be potentially used to prevent the early infection of EV71.

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