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Protocols

Applied biotechnology for production of immunoglobulin Y specific to hepatitis A virus

Vanessa Salete de Paula[a], Alexandre dos Santos da Silva[a], Gentil Arthur Lins Bentes Mendonça de Vasconcelos[a], Ezio Tavares Iff[b], Maria Eduarda Monteiro Silva[b], Livia Abud Kappel[a,b], Paula Borba Cruz[a], Marcelo Alves Pinto[a,b,*]

[a] Oswaldo Cruz Institute/FIOCRUZ, Rio de Janeiro, RJ, Brazil
[b] Veterinary Medicine School, C.C.S., UNIFESO, Teresópolis, RJ, Brazil

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A B S T R A C T

A new protocol for producing polyclonal antibody against hepatitis A virus (HAV) is described. Twenty hens were immunized three times with a commercial HAV vaccine and HAV from a cell culture with three types of adjuvants: CpG oligodeoxynucleotides (CpG-ODN), incomplete Freund's adjuvant and an alum adjuvant. In each of the last two booster inoculations, blood from the birds was collected and tested for HAV antibodies. Egg yolk was separated from egg white and immunoglobulin Y (IgY) antibody was then purified by polyethylene glycol 6000. The mean yield of total protein in yolk was 22.62 mg/mL. Specific activity of the antibody was tested using commercial ELISA, Western blotting, and *in vitro* neutralization assay demonstrating that anti-HAV IgY bound specifically. After the first immunization, birds immunized with HAV from cell culture plus incomplete Freund's adjuvant with/without CpG-ODN showed highest levels of anti-HAV IgY in serum ($p < 0.05$). Viral combination with CpG-ODN resulted in early response of anti-HAV serum in hens, reflecting the amount of IgY transferred to the egg yolk ($p < 0.05$). The results suggest that egg yolk may be a large scale source of specific antibodies against hepatitis A virus. Further applications of this method have yet to be tested.

1. Introduction

Hepatitis A virus (HAV) is a non-enveloped RNA virus of icosahedral particle with a diameter of 27–32 nm, family *Picornaviridae* and genus *Hepatovirus* (Hollinger and Emerson, 2001). HAV replicates in the liver and is excreted in faeces. Hepatitis A virus can remain stable in the environment for several months and are able to survive in freezing conditions (Hutin et al., 1999). Most of the world population is still under moderate to high risk of being infected by hepatitis A virus (Hendrickx et al., 2008).

Antibodies extracted from egg yolks have been used as an alternative to mammalian IgG for serological tests for *Helicobacter pylori* and hepatitis B virus (Cova, 2005). Immunoglobulin Y (IgY) antibodies represent the avian counterpart of mammalian IgG predominantly in the immunoglobulin fraction serum found of birds, reptiles and amphibians. Immunoglobulins are transferred from the plasma of dam into the egg yolk through a IgY receptor (FcRY) found in the yolk sac of birds thus providing passive immunity to the offspring (Patterson et al., 1962; Loeken and Roth, 1983). Comparatively, this role corresponds to placental or colostrums IgG transfer in mammals (Leslie and Clem, 1969).

Adjuvants are compounds that improve the immune response against co-inoculated antigens. Oligonucleotides containing dinucleotide (CpG) can enhance the production of antibodies by the agonistic effect on Toll-like receptor (TLR) 9 that leads to enhanced specific-antigen B-cell proliferation and differentiation, resulting in extra-follicular plasma cells (Vleugels et al., 2002; Wang et al., 2009). Synthetic oligonucleotide sequences containing CG (CpG-ODN) are a Pathogen Associated Molecular Patterns (PAMPs) and are interpreted as a signal of invasion by the innate immune system and activate subsequently defense patterns (Krieg et al., 1995; Heeg et al., 2008). Other adjuvants such as alum, incomplete as well as complete Freund's types have been described as potent inducers of immunogenicity due to a dependent TLR2 and TLR4 agonistic activity (Tsuji et al., 2000). Intracellular receptors, such as nucleotide-binding oligomerization receptors (NLRs) were associated with adjuvant immunomodulatory effects (Garlapati et al., 2009). Such receptors recognize muramylpeptides released both from bacterial wall and retinoic-acid-inducible protein 1 receptor

* Corresponding author at: Instituto Oswaldo Cruz, FIOCRUZ, Av. Brasil, 4365, Manguinhos, Pav. Hélio e Peggy Pereira, 2nd Floor, B221, ZIP Code: 21.045-900, Rio de Janeiro, RJ, Brazil. Tel.: +55 21 25621924; fax: +55 21 22706397.
E-mail address: marcelop@ioc.fiocruz.br (M.A. Pinto).

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(RIG-I-like receptor or RLRs) as an intracellular detector of single-stranded RNA of viral origin (Ferwerda et al., 2010).

The immune response of birds treated with HAV antigen combined with different adjuvant was compared to anti-HAV IgY titers both in samples of serum and egg yolk.

2. Materials and methods

2.1. Hens

Twenty healthy two-weeks old Isa Brown laying chickens were purchased from a commercial broiler supplier. They were then divided into five groups of four birds, each hen weighing between 0.143 kg and 0.220 kg. Animals were housed in the animal facilities at the School of Veterinary Medicine of Centro Universitário Serra dos Órgãos (UNIFESO, Teresópolis, Rio de Janeiro, Brazil). One week before the beginning of the experiment all hens were housed individually in appropriate cages, in a regimen of 12 h of light to 12 h of darkness, at room temperature around 24 ± 3 °C and relative humidity of 75 ± 5%. All chicks were vaccinated against Fowlpox virus and New Castle disease virus. Water and commercial food were offered *ad libitum* considering the life stage of each bird. Animals were checked on a weekly basis to monitor the weight gain. Blood was also collected (1 mL) from the wing vein to confirm the effectiveness of HAV immunization before the egg laying period. Hens were euthanized at 31 weeks-old, 16 weeks after the beginning of the egg laying period, under deep anesthesia. The experimental protocol was reviewed and approved by the Ethics Commission for the Use of Animals – CETA-UNIFESO protocol no. 0272/2009.

2.2. Antigen preparation and immunization schedule

Five different immunization protocols were carried out as followed: group I: 200 μL of commercial pediatric HAV vaccine (Merck & Co., West Point, PA, USA) was added to 20 μL CpG ODN (1 mg/mL) (5′-TCG TCG TTT GTC GTT TTG TT-3′, Integrate DNA Technologies, USA); group II: commercial pediatric HAV vaccine only; group III: 100 μL of HAV from culture cell (10^5 copies/mL) added to two types of adjuvant: incomplete Freund's adjuvant (100 μL) (Sigma–Aldrich, St Louis, MO, USA) and CpG-ODN (20 μL); group IV: 100 μL of HAV from culture cells added to incomplete Freund's adjuvant only (20 μL); and group V: incomplete Freund's adjuvant (100 μL) without virus or vaccine as the negative control. The HAV (HAF-203 Brazilian strain (Gaspar et al., 1993), accession number: GenBank AF268396) administrated in birds was produced in cell culture (FRhK-4) with 10^5 copies/mL. A total volume of 220 μL was administrated intramuscularly into the pectoral muscle. Such procedure was chosen as the route of immunization for all groups. The first immunization occurred when birds were 3-weeks-old; two further boosters were scheduled for the 7th and 11th week, respectively. Hens were weighed and bled (1 mL) via a wing vein before each immunization. Eggs were collected daily, beginning 30 days after the third immunization for 16 weeks and stored at 4 °C. Egg yolks were separated from the whites and stored at −20 °C. After this period, birds were euthanized by heart puncture and exsanguination, under deep anesthesia. The necropsy was performed in the Pathology Laboratory at the School of Veterinary Medicine of Centro Universitário Serra dos Órgãos, Teresópolis, Rio de Janeiro, Brazil. In this procedure, samples of liver, bursa of Fabricius, kidney and spleen were also collected, fixed in 10% buffer formalin and stored in paraffin blocks for further conventional histological analysis by optical microscopy.

2.3. Detection of anti-HAV IgY antibodies in serum by enzyme-linked immunosorbent assay

To confirm seroconversion, blood samples were collected and centrifuged at 8000 × *g* for 10 min at 4 °C. The serum was transferred to a 1.5 mL tube and stored at −20 °C. HAV specific IgY antibodies found in serum samples from immunized hens were detected by enzyme-linked immunosorbent assay (Biokit, Barcelona, Spain) according to the manufacturer's instructions.

2.4. IgY purification

Separation of IgY from egg yolk was performed using polyethylene glycol precipitation according to other investigators (Polson et al., 1980, 1985). One part of the egg yolk (10 mL) of the same immunized group of hens was mixed briefly with four parts (40 mL) of phosphate-buffered saline (PBS), pH 7.5. Egg yolks were treated with 3.5% polyethylene glycol (PEG) 6000 by adding it to the egg yolks and incubated at room temperature for 20 min. This procedure was then followed by centrifugation at 5000 × *g* for 25 min. After centrifugation, the precipitate was discarded. The supernatant phase was stirred with 12% PEG 6000 and then incubated for 10 min at room temperature, followed by a 25 min of centrifugation at 5000 × *g*. The supernatant was discarded and the precipitate was dissolved in original volume of PBS and precipitated again with 12% of PEG 6000. The mixture was centrifuged once again as described above and the precipitate was dissolved in half of the original yolk volume (5 mL) of PBS.

2.5. Characterization of IgY by SDS-PAGE and Western blotting

SDS-PAGE was performed using the Bio-Rad® Mini gel system under reduced and denatured conditions. HAV virus was loaded into 12% of Bis–Tris gel and was run at 200 V for 40 min until the front dye reached the bottom of the gel casing. Gels were stained with Comassie Blue stain to visualize proteins. Following SDS-PAGE, proteins were electrotransferred to nitrocellulose membrane in Western blotting transfer buffer by running at 100 V for 120 min. The membrane was blocked in PBS-T 0.05% and added to a solution of powder milk at 5% and left overnight at room temperature with agitation, then washed three times (5 min per wash) in PBS-Tween 0.05%. The membrane was incubated with IgY from each group (I–V) diluted 1:5000 in PBS-Tween 0.05%, in a solution of powder milk 5%, incubated at 37 °C for 4 h with agitation. After incubation, the membrane was washed as described above. Afterwards, membrane was incubated at 37 °C for 1 h with rabbit IgG anti-IgY (Sigma, Steinhein, Germany) 1:5000 in PBS-T 0.05. The membrane was washed and incubated with A protein (Sigma, St Louis, USA) added to diluted peroxides at 1:500 in PBS-T for 1 h at 37 °C with agitation; followed by a final wash, threefold with PBS-T and threefold with PBS. HAV protein bands were visualized using 5 mg of 3,3′-diaminobenzedine tetra hydrochloride in 20 μL of peroxides, 100 μL of $CoCl_2$ in 10 mL of PBS. The reaction was stopped by adding distilled water.

2.6. Characterization of anti HAV IgY antibodies in yolk by enzyme-linked immunosorbent assay

The purified IgY anti-HAV were diluted serially (10-folds) for evaluating antibody activity and to compare the titration of antibody levels using CpG-ODN as adjuvant. HAV specific anti-HAV IgY from immunized hens was measured by enzyme-linked immunosorbent assay (Biokit, Barcelona, Spain) according to the manufacturer's instructions. Absorbance was recorded using an ELISA plate reader. IgY of eggs collected from non immunized hens was used as a negative control.

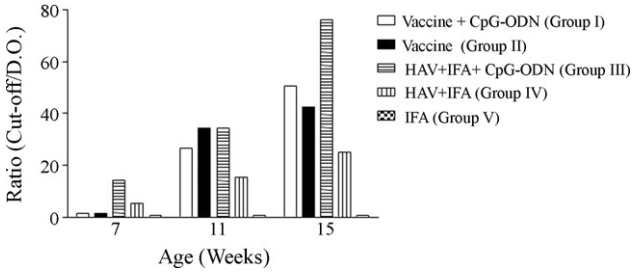

Fig. 1. Antibody responses from differently immunized groups. Each bar in the graph represents the average anti-HAV IgY found in the serum of four hens. Immunization procedures using the same antigen were performed at 4-week intervals under identical conditions. The comparative analysis of anti-HAV IgY titers in different groups showed the highest antibody titers in groups III and IV at 30 days after the first immunization ($p < 0.001$) or seven weeks age.

2.7. In vitro neutralization of hepatitis A virus by anti-HAV IgY

In order to evaluate antibody specificity of IgY-neutralizing activity to HAV, an assay was performed in cell culture. Serially diluted IgY (1:1, 1:10, 1:100, 1:1000, and 1:10000) were incubated with 10^5 copies/mL of HAV at 37 °C for 1 h. The inocula which were adapted previously to FRHK-4 culture cell were added to monolayers and kept for 10 days at 37 °C with 2% CO_2. The replication of HAV was estimated by a quantified real-time PCR technique for detecting negative intermediate RNA as described previously by de Paula et al. (2009).

2.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5.0 for Windows Inc. Data were reported as means ± standard deviation (SD) and levels of significance were evaluated using ANOVA. Differences were considered significant at level of $p < 0.05$.

3. Results

3.1. Effects of immunization on the biology of hens

All immunized hens remained healthy throughout the experiment. The injection sites were painless at palpation, causing neither discomfort to the animals nor any tissue damage. No abnormality in the development of the hens was observed during the immunization period. A common feature observed in all groups was a subclinical fatty change determined by loss in the liver architecture by disruption; some areas of hepatic congestion were also detected. Congestion in the spleen was the principal change detected in all groups. Kidneys and Bursa of Fabricius sections presented normal architecture by disruption.

3.2. Characterization of specific anti-HAV IgY in sera and yolk

Comparative analysis of serum titer of anti-HAV IgY in different immunization groups showed the highest antibody titers in groups III and IV at 30 days after the first immunization ($p < 0.001$) which corresponds to seven weeks of life of animal. In the second and third months following post-immunization, no statistical differences were detected between groups (Fig. 1). When anti-HAV IgY was titrated in yolk in each group, group III also showed high titer of anti-HAV IgY (Fig. 2). However, no significant difference was observed among groups ($p > 0.05$). Then hens began laying period at 15 weeks of life (30 days after the third immunization); all 1406 eggs were collected for 16 weeks. After extraction of yolk protein by polyethylene glycol 6000, the total protein concentration was measured per group; the mean yield of protein was 22.62 mg/mL.

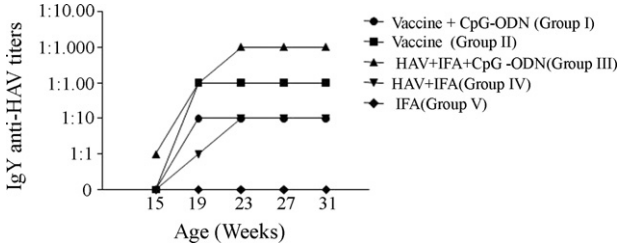

Fig. 2. Representation of mean specific anti-HAV IgY titers found in egg yolk of each immunized group of hens. Anti-HAV titers were not detected in IgY from negative control group.

SDS-PAGE was applied to confirm isolation of immunoglobulin. The electrophoresis pattern of gel filtration found in this procedure was in accordance with the standard IgY; showing 68 kDa heavy chain and 27 kDa light chain (Fig. 3). The presence of anti-HAV IgY in egg yolks was confirmed by Western blotting (Fig. 4) and ELISA. Groups I and III, inoculated with CpG-ODN, showed the stronger bands by Western blotting. IgY obtained from hens immunized with and without HAV were tested by ELISA for quantifying the specific anti-HAV antibodies in egg yolks. The ELISA titers ranged from 1:10 to 1:10,000 according to the group; group III, immunized with CpG-ODN, HAV and incomplete Freund's adjuvant, showed high anti-HAV IgY titers in the yolks at 23–31 weeks.

To confirm the specificity of anti-HAV IgY in blocking HAV penetration in FRhK-4 cells, an *in vitro* neutralization assay using HAV was performed. Results showed that antibodies from yolks were proven effective when neutralizing the virus up to 1:100 dilutions. A small amount of HAV replication began at 1:1000 dilutions, 65 copies/mL.

4. Discussion

Chickens, as a source of desired antibodies, represent an alternate animal system that offers some advantages with respect to animal care, high productivity and special suitability of avian antibodies for certain diagnostic purposes. Despite being an excellent counterpart to mammal IgG, chicken IgY antibodies still represent an underused resource. This may be due to the lack of informa-

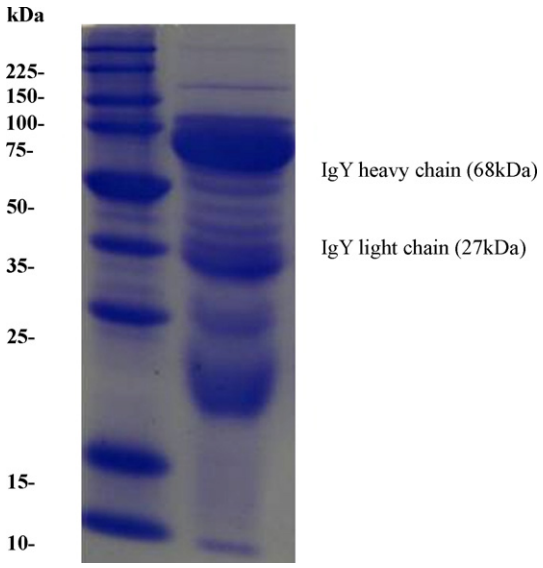

Fig. 3. Sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) gel of purified immunoglobulin (Ig)Y from egg yolk. The acrylamide concentration was 12%. Lane 1 – molecular weight, lane 2 – IgY.

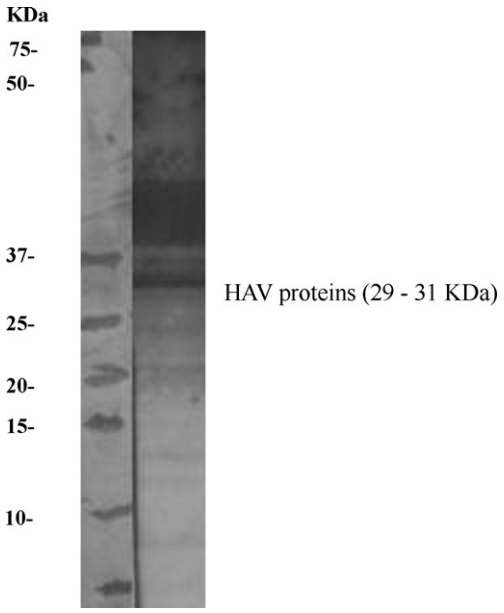

Fig. 4. Western Blot analysis of IgY anti-HAV. Hepatitis A virus was incubated with IgY anti-HAV. HAV was incubated with IgY anti-HAV. Lane 1 – molecular weight; lane 2 – HAV proteins.

tion concerning the possibility of production and application of IgY or their use is being hampered by problems with keeping the chickens and with IgY isolation (Narat, 2003). The immunoglobulin against HAV used in diagnostic assays is mainly polyclonal antibodies derived from human plasma. This study compared the antibody response of the hens immunized with the vaccine and HAV with CpG ODN as adjuvant to produce specific IgY against HAV in yolk egg. During the study 1406 eggs were obtained in 16 weeks, with average protein concentration of 22.62 mg/mL. Previous studies reported that the content of total IgY in egg yolks is relatively constant, with an average of 0.6%, wt/wt, between the hens regardless of breed, egg weight and ability to lay eggs (Li et al., 1998). Similar results have been reported previously regarding the concentration of IgY in egg yolks during immunization of hens (Shimizu et al., 1988; Sunwoo et al., 1996). The beginning of the period of egg laying happened at week 15 with no specific relevant clinical abnormalities and normal growth curves of hens formation of liver fat. In this study, yield of anti-HAV IgY production was assessed based on HAV immunization of hens when using different protocols.

To avoid reduction of the egg laying activity in young birds (Levesque et al., 2007), the first immunization was performed when hens were 3-weeks-old, and the last immunization was performed 30 days before the period of egg laying. At 15 weeks, low titers of anti-HAV IgY were detected in yolks (Fig. 2) suggesting that titers of anti-HAV IgY are transferred slowly from serum to yolk with the highest titer at week 23 (Fig. 2) as described previously (Loeken and Roth, 1983). Hens immunized with HAV plus incomplete Freund's adjuvant with/or without CpG-ODN showed a more rapid serological immune response to HAV just after the first immunization. After the second immunization, all groups seroconverted. Interestingly, when anti-HAV IgY were quantified in yolks, birds of group III showed an early transference of anti-HAV IgY to yolk. Groups immunized with commercially available vaccines with or without CpG-ODN (group I and II) also reached a high titer at 15-weeks-old, confirming that commercial vaccines may be useful to production of anti-HAV IgY.

The specificity of IgY to hepatitis A virus was confirmed by ELISA and Western blotting. The electrophoretic pattern of gel filtration was in accordance with the standard IgY (Devi et al., 2002). Although the antibody titers are variable according to formulation of the inoculums used for each immunization, the amount of antibodies produced was consistently high. Each group laid approximately 280 eggs during a period of four months and a single egg yolk contains 100–150 mg of IgY antibodies resulting in 28–42 g of IgY per chicken/year (Mine and Kovacs-Nolan, 2002; Pauly et al., 2009). The antigen-specific IgY antibodies represented between 2 and 10% of the total IgY obtained (Schade et al., 1994). The results of specific anti-HAV IgY were pooled at each sampling time for each adjuvant system tested. In group III, the anti-HAV IgY in eggs reached a first peak at week 23 and stabilized for at least 31 weeks onwards. When compared with the use of incomplete Freund's adjuvant and HAV (group IV), incomplete Freund's adjuvant supplemented with CpG-ODN (group III) showed an increase of 100-fold titers of anti-HAV IgY during the whole experiment. The mean specific anti-HAV IgY concentration of group III was the highest from the first week onwards. This difference was significant ($p < 0.05$). According to Levesque et al. (2007), supplementation of incomplete Freund's adjuvant with CpG-ODN resulted in a large and stable augmentation in the titer of specific IgY present in eggs. The yield of specific IgY increased with time, so antibody recovery remained high, even after prolonged immunization periods. Furthermore, the IgY showed neutralizing capacity when used to block the replication activity of the HAV *in vitro*.

In this study, the aim was to try to enhance anti-HAV IgY immune response by co-administration of incomplete Freund's adjuvant with synthetic oligodeoxynucleotide. CpG-ODN are synthetic agonists of Toll-like receptor 9 and potent inducers of innate and acquired immunity (Vincent et al., 2009). It has been shown that CpG-ODN can function as an effective adjuvant for vaccines against a variety of pathogens such as bacteria, viruses, fungi and parasites (Davis et al., 1998; Moldoveanu et al., 1998; Eastcott et al., 2001; Mahmood et al., 2006). As for HAV, CpG-ODN has been found beneficial for increasing the immunogenicity of inactivated HAV vaccine and virus. This study found that the addition of CpG-ODN as adjuvant was able to enhance IgY-specific immunity in hens, indicated by elevated IgY antibody titers in serum and egg yolks. The addition of CpG-ODN as shown in Figs. 1 and 2, enhanced significantly anti-HAV IgY. Regarding animal welfare regulations, the immunization of hens offered advantages such as immunization with CpG-ODN as adjuvant was well tolerated.

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