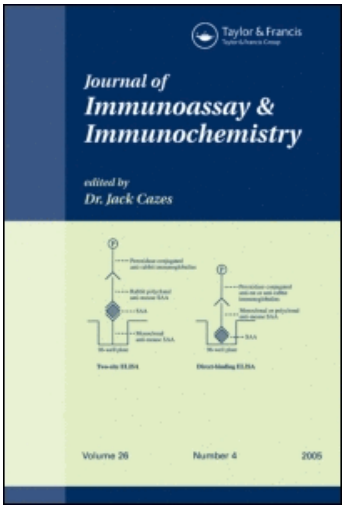

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Abstract: HLA-A*0201 α chain and β2m were expressed from a prokaryotic system, and after refolding and purification, the α chain and β2m were used to immunize eight laying hens. The titer of egg yolk antibody against α chain increased from 10^2 to $10^{5.3}$. The titer of egg yolk antibody against β2m increased from 10^1 to $10^{4.7}$. The extent of titer increase is similar between the two antigens. An average of 135 mg purified polyclonal antibody (IgY) can be easily obtained from one egg yolk. The use of egg collection rather than serum collection is compatible with modern animal protection regulations. An average of 28 eggs were obtained from a laying hen every month, with a total amount of 3780 mg immunoglobulin extracted from one immunized hen

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every month, which would be equivalent to 630 mL of serum or 1260 mL of blood per month. Chickens are an optimal host for the production of polyclonal antibodies with high titer and high yield. Purified IgY was labeled with horseradish peroxidase and reacted with PBMC on nitrocellulose membranes indicating that the antibody can bind to the native conformation of class I HLA molecule on PBMC.

Keywords: Quantification, Titer, HLA-A*0201 α chain, β2-Microglobulin, IgY, Immunization, Cell surface molecules

INTRODUCTION

Traditionally, the production of antiserum from the blood of immunized mammals is commonly used. Like mammals, birds protect their offspring by transferring maternal antibodies from serum to egg yolk.[1,2] It has been noted that the levels of immunoglobulin in the yolk is as high as, or even greater than, those in chicken serum.[3] The properties of the IgG from avians are slightly different from those of mammalian IgG, and thus, these molecules are called IgY. The molecular mass of IgY is 180 kDa, which consists of two subunits: a heavy chain of 67 kDa–70 kDa and a light chain of 22 kDa–30 kDa.[4]

The use of hen egg yolk as a source of specific antibodies offers considerable advantages, including the rapid production of large volumes of highly concentrated egg yolk containing IgY.[5] In addition, the collection of eggs is compatible with modern animal protection regulations, and is cost effective and convenient.

HLA molecules exist on the surface of almost all nucleated cells and consist of two separate polypeptide chains. The alpha chain is an MHC-encoded, transmembrane molecule containing three extracelluar domains α1, α2, and α3, while the β chain is a non-MHC encoded small protein called β2m with a molecular mass of 12 kDa. Because birds are known to produce antibodies against highly conserved proteins of mammals,[6–8] chickens were immunized with HLA-A*0201 α chain and β2m. Dot blot analysis showed that antibodies against HLA-A*0201 α chain and β2m can bind to the native conformation of the HLA-I class molecule on the surface of PBMC.

EXPERIMENTAL

Preparation of Purified α Chain and β2m

HLA-A*0201 α chain and β2m were expressed in the form of inclusion bodies from prokaryotic systems. Briefly, the inclusion body was suspended in washing solution (20 mM Tris-HCl, 2 mM EDTA, 2 M Urea, 0.1% Triton X-100, 2 mM mercaptoethanol, pH 8.0) and stirred evenly for 20 min, followed by centrifugation 25,000 g for 15 min at 4°C and then repeating

the above steps. After washing, the inclusion body was solubilized in 7 M guanidine hydrochloride buffer (containing 50 mM Tris-HCl, 100 mM mercaptoethanol, 1 mM EDTA, pH 8.0). Then 20 mM Tris-HCl (pH 8.0) was added to the solution until the white precipitate appeared. After centrifugation, the precipitate was solubilized in 8 M urea solution (containing 20 mM Tris-HCl, 100 mM mercaptoethanol, 1 mM EDTA, pH 8.0). Then, the urea solution of the inclusion body was added to the refolding buffer (20 mM Tris-HCl, 0.1 mM oxidized glutathione, 1 mM reduced glutathione, 1 mM EDTA, pH 8.0) and the final protein concentration was adjusted to 0.2 mg/mL. The refolded α chain and β2 m were purified by ion exchange chromatography on a DEAE Sepharose Fast Flow column equilibrated in 20 mM Tris-HCl (pH 8.0). The alpha chain was not absorbed while β2 m was eluted by 0.1 M NaCl. The purified α chain and β2 m proteins were dialyzed against PBS at 4°C, and then were used to immunize laying hens.

Immunization of Laying Hens

Eight white laying hens were named A1, A2, A3, A4, B1, B2, B3, and B4.

On day 0, A1, A2 received a first injection with 200 μg α chain emulsified with an equal volume of complete Freund's adjuvant. A3, A4 were immunized the same as A1, A2 but each injection also contained 20 μg CPG. According to reports, CPG can reinforce immune reaction.[9–12] B1, B2 received a first injection with 200 μg β2m, emulsified with an equal volume of complete Freund's adjuvant. B3, B4 were immunized the same as B1, B2 but each injection also contained 20 μg CPG. The emulsified antigens were injected into cervical subcutaneous part of each bird.

On day 14, a second injection was similar to the first injection, except that the adjuvant was Freund's incomplete adjuvant and the injection position was intramuscularly under the wing.

On the 28th day, A1, A2, A3, A4 received a third injection with 200 μg α chain without adjuvant. B1, B2, B3, B4 received a third injection with 200 μg β2m without adjuvant. The injection position was intramuscularly under the wing.

On 56th and 84th days, the eight hens were given a fourth and fifth injection identical to the third injection.

The eggs were collected daily, marked, and stored at 4°C until use.

Estimation of the Titer of IgY by Enzyme-Immunoassay (ELISA)

The purified antigen was used to coat microtiter plate wells at 0.5 μg/well. Following overnight incubation at 4°C, the plates were washed three times by PBST. The different diluted samples of 100 μL were added to the wells and incubated for 1 h at 37°C. A secondary antibody conjugate, a mouse anti-chicken IgG HRP conjugate, was added at a concentration of 1:3000

and incubated for 1 h at 37°C. The wells were washed three times and the substrate for the enzyme detection was 3,3′,5,5′-tetramethylbenzidine(TMB). Specific antibody activity was investigated before and after immunization of the eight laying hens.

Extraction and Purification of IgY

IgY was purified from the egg yolks as described with minor modifications. Briefly, the egg yolk was diluted with two vols. of PBS (pH 7.4). The pH of the diluted egg yolk was adjusted to 4.6 with acetic acid and caprylic acid was slowly added dropwise at 0.6 mL/min, to give a final caprylic acid concentration of 64 μL/mL egg yolk dilution. The mixture was stirred at room temperature for 2 h, centrifuged at 20,000 g for 10 min at 4°C, and filtered to remove the bright yellow oil layer on the surface. The pH of the extract was adjusted to 7.4 with 1 M Tris and centrifuged at 20,000 g for 10 min at 4°C. The preparation was cooled to 4°C and ammonium sulfate was added to 0.231 g/mL solution, allowed to stir at 4°C for 1 h and centrifuged at 20,000 g for 10 min at 4°C. The pellet was dissolved in PBS (pH 7.4) to a volume equal to that of the undiluted harvested egg yolk and dialyzed against PBS to remove ammonium sulfate.

Detection of Class I HLA Molecule on the Surface of PBMC, T2, and K562 Cells

PBMC, T2 cells, and K562 cells were resuspended in 100 μL FB (2% FCS and 0.1% sodium azide in PBS). Then the suspensions were aliquoted to two tubes (50 μL/tube). To one tube, was added 1 μL FITC-conjugated W6/32, an antibody that recognizes the integral conformations of class I molecule. To the other tube, was added 1 μL FITC-conjugated anti-β2m. After incubation at 4°C in the dark for 1 h, the cells were washed twice with cold FB and fixed in PBS containing 2% formaldehyde before they were subjected to FACS analysis.

IgY Labeled with Horseradish Peroxidase

HRP (10 mg) was dissolved in 1 mL HAC-NaAC buffer (pH 5.6) and added to 0.5 mL $NaIO_4$ (0.1 M), and reacted at 4°C for 30 min. Glycol, 1 mL (2.5%) was added and incubated at room temperature for 30 min. Then 10–20 mg antibody was added to the enzyme buffer. The pH of the buffer was adjusted to 9.0 with 0.05 M CBS (pH 9.5) and the mixture remained at 4°C overnight. On the second day, 0.2 mL $NaBH_4$ (5 mg/mL) was added to it and reacted at 4°C for 2 hours. After dialysis against PBS, antibodies labeled with HRP were aliquoted and frozen until further use.

Dot Blots Analysis of the IgY Reaction with PBMC

PBMC of 1, 2, 3 μL (10^7/mL) were spotted onto a NC membrane. At the same time, parallel studies were done using the T2 cells and K562 cells. It is known that on the surface, T2 cells had a low expression of class I HLA molecule, and K562 cells did not express class I HLA molecule. The membrane was blocked for 15 min at 37°C with 2% non-fat milk in PBST, and then incubated with the IgY labeled with HRP at 37°C for 30 min. Following three washes with PBST, binding was revealed by incubation with a diaminobenzidine reagent.

RESULTS

Titer Change of IgY

Chickens were immunized five times. After the first three immunizations, the levels of specific antibody activity increased. After the fourth and fifth immunizations, the titers of IgY no longer increased, instead, slightly decreased. The high titers of antibody level remained in the fifth month after immunization.

After immunization, the titer of IgY from eight hens increased greatly (Figure 1). The titer of egg yolk antibody against α chain increased from 10^2 to $10^{5.3}$. The titer of egg yolk antibody against β2m increased from 10^1 to $10^{4.7}$. A3, A4, B3, B4 were immunized with the addition of CPG adjuvant, but the titers of IgY were not higher than that of A1, A2, B1, B2. This preliminarily suggested the immune effect of Freund's in combination with CPG adjuvant is not more powerful than Freund's alone on chickens.

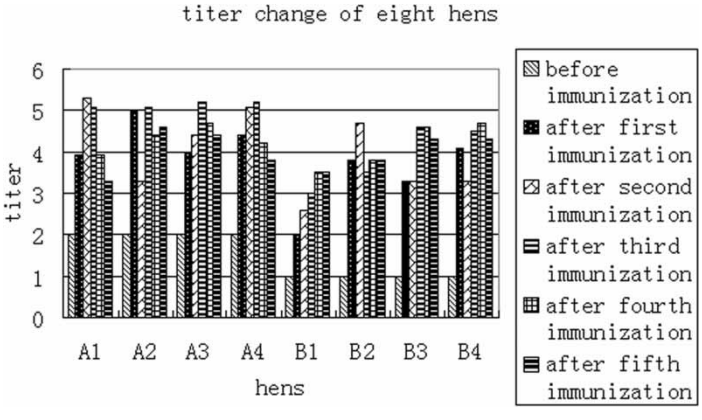

Figure 1. Titer change of eight hens. The titer is shown as 10^n. After immunization, the titer of IgY from eight hens increased greatly. The highest titer of egg yolk antibody against α chain is $10^{5.3}$. The highest titer of egg yolk antibody against β2m is $10^{4.7}$.

Because the sample numbers is not enough, we cannot conclude that CPG has no effect on reinforcing immune reactions.

SDS-PAGE Analysis of Antigens and Antibodies

After purification, the molecular mass of HLA-A*0201 α chain is about 32 kDa, and of β2m is about 12 kDa, which is in agreement with documented reports. The purified IgY has two major bands, the molecular mass of the heavy chain is about 66 kDa, and of the light chain is about 25 kDa (Figure 2).

Detection of Class I HLA Molecule on the Surface of PBMC, T2 and K562 Cells

The results of FACS analysis showed that on the surface of PBMC there was a high expression of class I HLA molecule, while T2 cells had a low expression of class I HLA molecule, and K562 cells did not express class I HLA molecule (Figure 3).

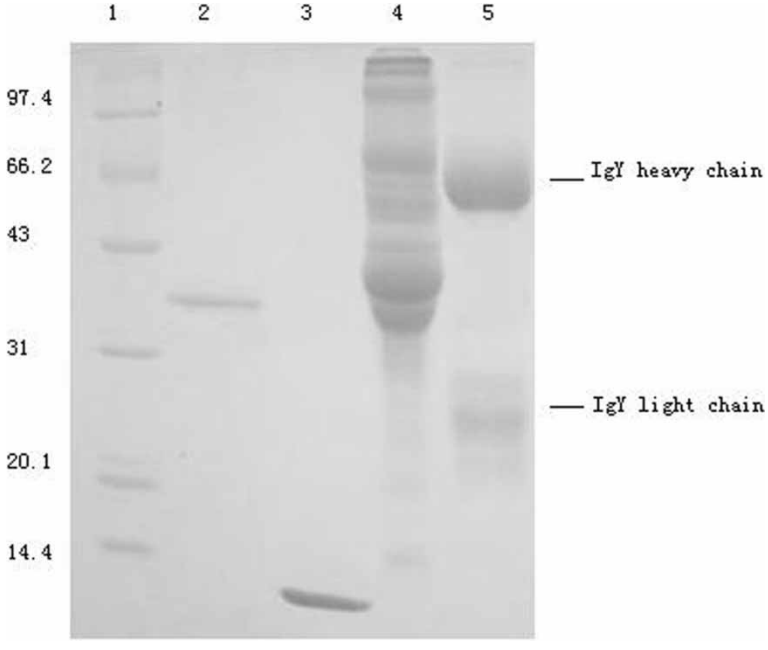

Figure 2. SDS-PAGE analysis of antigen and egg yolk antibody. Lane 1: standard proteins sized in kDa; lane 2: purified HLA-A*0201 α chain; lane 3: purified β2m; lane 4: IgY before purification; lane 5: IgY after purification.

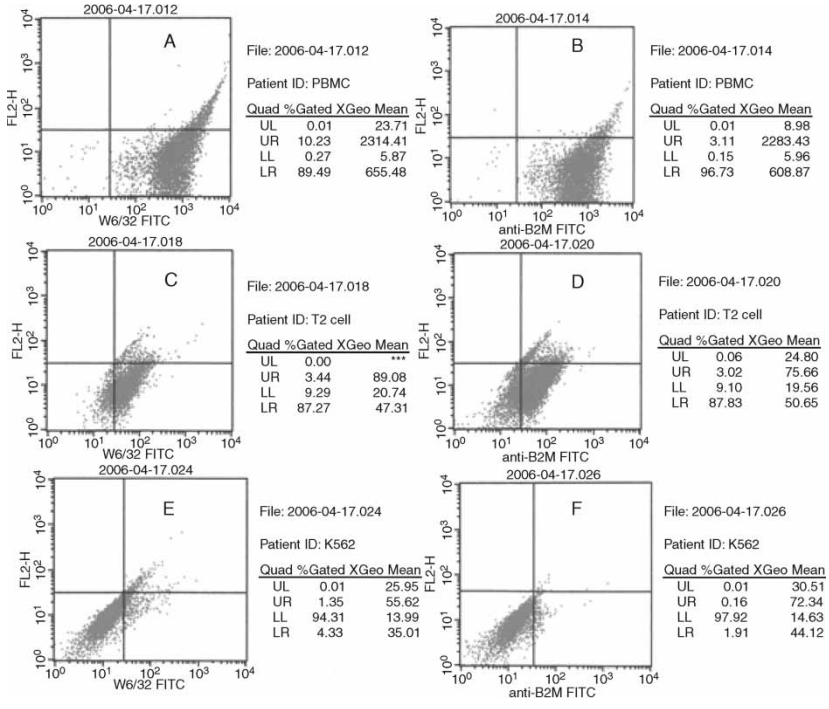

Figure 3. Detection of HLA class-I molecules on the surface of PBMC, T2 and K562 cells. A. PBMC + 1 μL W6/32-FITC. B. PBMC + 1 μL anti-β2m-FITC. C. T2 cells + 1 μL W6/32-FITC. D. T2 cells + 1 μL anti-β2m-FITC. E. K562 cells + 1 μL W6/32-FITC. F. K562 cells + 1 μL anti-β2m-FITC.

Dot-Immunobinding Assay

IgY against α chain and β2m can react with the HLA I class molecule on the surface of PBMC, as shown in the positive immunoblot. The controls of T2 and K562 cells, which have low or no HLA I class molecule conformation on the surface are negative as expected (Figure 4).

IgY Yield

A laying hen usually lays an egg every day. An average of 28 eggs will be obtained from a hen per month. An egg contains 15 mL egg yolk on the average, and approximately 9 mg of purified IgY can be obtained from 1 mL egg yolk using our purification method. So, 135 mg IgY can be obtained from an egg. A laying hen can produce 3780 mg IgY per month, which may amount to the equivalent of 630 mL of 6 mg/mL of serum, or 1260 mL of blood. The amount of purified IgY produced in a chicken is far

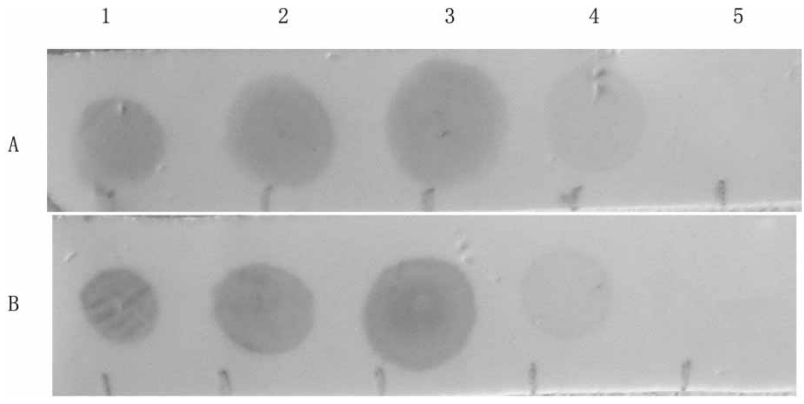

Figure 4. Dot blots analysis of cell surface molecule. Lane 1: 1 μL PBMC; lane 2: 2 μL PBMC; lane3: 3 μL PBMC; lane 4: 1 μL T2 cells; lane 5: 1 μL K562 cells. A: incubation with IgY against α chain labeled with HRP; B: incubation with IgY against β2m labeled with HRP. The cell concentration is 10^7/mL.

higher than that of IgG produced in a rabbit (Table 1). Only 60 mL blood can be drawn form a rabbit.

DISCUSSION

Chickens can produce high titer antibodies against the antigen on the surface of human nucleated cells. HLA-A*0201 α chain and β2m were expressed from prokaryotic systems. After refolding and purification, they were used as antigens to immunize laying hens. The titer of egg yolk antibody against α chain increased from 10^2 to $10^{5.3}$. The titer of egg yolk antibody against β2m increased from 10^1 to $10^{4.7}$. The extent of titer increase is similar between two antigens. The high titer antibody will be present in egg yolks for five months after immunization of the chicken, thus ensuring the IgY yield. The titer change figures showed three times immunizations is enough to produce IgY. The fourth and fifth immunizations did not appear to be essential. After the second immunization, high titer antibodies can be obtained and will persist for

Table 1. IgY yield

mg IgY/mL egg yolk	mL Egg yolk/egg	eggs/ month	mg IgY/ egg	mg IgY/ month
9 ± 2	15 ± 4	28 ± 2	135 ± 20	3780 ± 300

Values are means $\pm$ standard deviations (n = 5).

five months. According to literature, CPG can reinforce immune reactions.[9–12] In our experiments, the immune effect of Freund's in combination with CPG adjuvant, is not more powerful than Freund's alone on chickens. Because the sample numbers are not enough, we cannot conclude that CPG has no effect on reinforcing immune reactions.

The IgY yield is extremely high. The IgY concentration in chicken serum is approximately 5–7 mg/mL.[13–16] In our experiments, a laying hen can produce 3780 mg IgY per month, therefore 3.78 g of egg IgY correspond, approximately, to the IgY content of 630 mL of serum or 1260 mL of blood. Only large mammals can produce comparable amounts of serum antibodies.

The collection of IgY is very convenient. One only needs to collect eggs every day and mark them and store in 4°C. While other mammals, such as rabbit and sheep, need to be bled. After bleeding, the animals will have discomfort and depression. The production of IgY lessened injury of the animals; there is no need for bleeding, and the laying rate is not affected. This is compatible with modern animal protection regulations.

The high purity and high activity of IgY can be obtained by using our purification method. The purity of IgY after purification is more than 90%. The IgY yield is 9 mg/mL egg yolk on average, using this method. The polyclonal antibody can recognize the native conformation of the HLA- I class molecule on the surface of PBMC. PBMC was spotted onto a NC membrane. The membrane was incubated with the IgY labeled with HRP. Binding was revealed by incubation with a diaminobenzidine reagent. An obvious dot formed. Instead, the position of T2 and K562 cells did not form dots because their surfaces don't have enough HLA- I class molecule conformation.[17–20] These cells stayed in 4°C refrigerators in PBS for five months, and then we repeated the experiments. The results of dot blots are similar to fresh cells. It indicated HLA- I class molecule conformation still existed after the cells died.

In conclusion, the IgY can react with the HLA- I class molecule on the cell surface. Cell surface molecules can be detected after cells had died for five months.

ABBREVIATIONS

IgY, immunoglobulin Y; ELISA, enzyme-linked immunosorbent assay; MHC, major histocompatibility complex; HLA, human leucocyte antigen; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline, pH 7.4; PBMC, peripheral blood mononuclear cells; HRP, horseradish peroxidase; β2m, β2-microglobulin.

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