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Iron-induced Chelation Alleviates the Potential Allergenicity of Ovotransferrin in a BALB/c Mouse Model

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Abbreviations

OVT, ovotransferrin;

Apo-OVT, iron-free OVT;

Holo-OVT, iron-chelated OVT;

Th, T helper;

OVM, ovomucoid;

OVA, ovalbumin;

Lys, lysozyme;

YGP42, yolk glycoprotein 42;

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis;

CT, cholera toxin;

ELISA , enzyme linked immunosorbent assay;

IgE, Immunoglobulin E;

IgG, Immunoglobulin G;

OD, optical density;

DCs, dendritic cells;

mMCP-1, mouse mast cell protease-1;

IL, interleukin;

IFN- γ , interferon gamma;

FITC, fluorescein isothiocyanate.

Abstract:

Ovotransferrin (OVT) is one of the main egg allergens with two iron-binding sites. Several studies have demonstrated that iron-chelation decreased the allergenicity of milk allergen and birch pollen allergens. Therefore, we hypothesized that iron-chelation could also reduce the allergenicity of OVT. Apo-OVT (iron-free OVT, the natural state in egg white) and Holo-OVT (iron-chelated OVT) were prepared, and the allergenicity of them were assessed and compared using a BALB/c mouse model as well as dendritic cells (DCs) based on antigen uptake. Mice were orally sensitized with Apo-OVT or Holo-OVT using cholera toxin as adjuvant. Clinical signs of allergy, morphological structure of jejunum, specific antibody levels, mast cell protease-1 (mMCP-1) concentrations, cytokines and antigen uptake by DCs were determined after the mice were challenged with Apo-OVT or Holo-OVT. Results showed that both Apo-OVT and Holo-OVT induced intestinal allergy, but no systematic allergic symbols were observed. Serum levels of mouse mast cell protease-1 (mMCP-1) and specific IgE in Apo-OVT group were lower than in control group, and no significant difference between Apo-OVT group and Holo-OVT group ($p>0.05$). The levels of OVT-specific IgG and IgG1, as well as the Th-1 cytokine interferon gamma and Th2-type cytokine interleukin-13 in Holo-OVT sensitized mice were significantly decreased compared to Apo-OVT group ($p<0.05$), while no significant difference with control group ($p>0.05$). However, DCs took in less Apo-OVT than Holo-OVT. Overall, iron-induced chelation could alleviate the potential allergenicity of OVT *in vivo*.

Keywords: egg allergen; ovotransferrin; mouse model; iron chelation; food allergy.

1. Introduction

Egg is one of the top 8 most allergenic foods to induce T helper 2 (Th2) allergic immune responses in children and infants [1-4]. Egg contains 10-12% of proteins, and four proteins in egg white, ovomucoid (OVM), ovalbumin (OVA), ovotransferrin (OVT) and lysozyme (Lys), and two proteins in egg yolk, a-livetin and yolk glycoprotein 42 (YGP42), were recognized as the allergens in egg [5-6]. Among these allergens, OVT accounts for 12% of egg white protein and is recognized by 22% of egg-allergic patients [7]. However, allergenicity assessment of OVT is somewhat limited compared to the extensive studies on OVM and OVA.

OVT is well known as an iron-binding monomeric glycoprotein with the function of binding and transporting iron ions [8]. It has two globular lobes, each containing an iron-binding site located within the inter-domain cleft of each lobe, allowing it to be easily chelated with the iron ions in solution [9], while OVT in egg white is normally without ferric ions [6]. Metal ions were demonstrated to change the structure and allergenicity of some allergens [10-13], and it was shown that iron-chelated OVT (Holo-OVT) had a different structure compared with iron-free OVT (Apo-OVT) [14-15]. Accordingly, it led to a puzzle about whether the iron-induced chelation had an effect on OVT allergenicity. In this study, we hypothesized that iron-chelation could decrease the allergenicity of OVT. Therefore, the study was designed to assess and compare the allergenicity of Apo-OVT and Holo-OVT by *in vivo* and *in vitro* methods. *In vivo* immune response to Apo-OVT and Holo-OVT was tested by allergic clinical

signs and pathological examination of intestine, mast cell protease concentration, and specific antibodies as IgG, IgG1 and IgE. In addition, cytokine production in a BALB/c mouse model, and *in vitro* immune response was tested with antigen uptake by dendritic cells (DCs).

2. Methods and materials

2.1. Purification of Apo-OVT

Apo-OVT was prepared from hen egg white according to our previous work [16]. Briefly, fresh hen egg white was diluted with two volumes of distilled water, followed by adjusting pH to 6.0 with 2 mol/L hydrochloric acid. The mixture was gently stirred for 3.5 h at 4°C enabling ovomucin precipitation. Afterward, the precipitates were removed by centrifugation at 3,000g for 5 min, followed by collecting the supernatant. The supernatant was adjusted to pH 8.0 with 3 mol/L sodium hydroxide, followed by centrifugation at 10,000g for 20 min at 4°C to remove insoluble materials. The supernatant was loaded onto a DEAE-Sepharose Fast Flow column previously equilibrated with 0.05 mol/L Tris-HCl buffer (pH 8.0) at a rate of 1.5 mL/min. Protein were eluted by linear gradients using 0-0.5 mol/L sodium chloride in 0.05 mol/L Tris-HCl buffer (pH 8.0) at a rate of 1.0 mL/min, and then fractionated and identified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The purity of prepared Apo-OVT was higher than 97%.

2.2. Preparation of Holo-OVT

Holo-OVT was produced from Apo-OVT with a modified method of Williams et al.[8].

Firstly, Apo-OVT was desalted by dialysis against distilled water at 4°C for 48h, followed by adjusting the concentration to 10mg/mL. Apo-OVT was then incubated at 4°C for 24h with 10-fold molar concentration of Fe^{3+} -nitrilotriacetic acid solution and 25 mmol/L of NaHCO_3 at pH 7.4. At last, the free Fe^{3+} was removed by ultrafiltration. The purity of Holo-OVT was calculated by Quality One software (Bio-Rad, USA).

2.3. Urea gel electrophoresis analysis

A novel urea gel electrophoresis was developed by our laboratory to identify Apo-OVT and Holo-OVT based on the method of Williams et al.[8]. Briefly, urea gel electrophoresis was carried out by using 8% urea-TBE gels with TBE buffer as the mobile phase, which was constituted by 112.5 mmol/L of Tris-borate solution and 250 mmol/L of EDTA (TBE). The urea gel was made up by 1.4 mL 30% Acryl amide-0.8% Bis Solution (37.5:1), 2.1 g urea, 0.7 mL 8×TBE, 5 μL N,N,N',N'-yteramethyl ethylenediamine, and 25 μL 10% Ammonium persulfate followed by adding H_2O to 7 mL. Samples were diluted in sample buffer containing 10% (v/v) 8×TBE, 6 mol/L urea, 50% (w/v) sucrose, and 0.2% (w/v) bromophenol blue. The parameters for urea gel electrophoresis were carried out at 150V for 4 h at 4°C.

2.4. Sensitization in a mouse model

The mouse model used in this study was similar to previously described work [17-18] with some modification. A total of 24 female BALB/c mice (6-8 weeks of age) were purchased from the Laboratory Animal Center of Nanchang University (Nanchang, China; permission number “SYXK (Gan) 2015-0001”). They were fed with an egg-free diet provided by Laboratory Animal Center of Nanchang University, which contained corn, rice, soybean, fish powder, and so on. All the mice had access to water and food ad libitum and were randomly divided into three groups (n=8/group). The mice were orally sensitized twice per week for 4 weeks with 1mg of Apo-OVT or Holo-OVT adjuvanted with 10 μ g of cholera toxin B subunit in 100 μ L solution, as outlined in Fig.1. The control group received distilled water instead of protein. All mice were orally challenged with 20 mg of OVT, followed by monitoring the clinical signs of allergy for approximately 30 min after the challenge by three independent and blind observers according to a scoring system as follows: 0 = no signs; 1 = scratching and rubbing around the snout and head; 2 = puffiness around the eyes and snout, diarrhea, piloerect, reduced activity; 3 = wheezing, labored respiration, cyanosis around the mouth and the tail; 4 = tremor and convulsions; and 5 = death. 40 min after the challenge, the mice were killed by cervical dislocation, and blood samples and spleens were collected for the measurement of various biomarkers. All animal-use protocols were approved by the Animal Care Committee of Nanchang University (permission number SYXK (Gan) 2009-003) under the guidelines of the Care and Use of Laboratory Animals (NIH Publication No. 86-23, Revised 1996).

2.5. Pathological examination of intestine

Regarding to the assessment of pathologic alterations, jejunum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin (H and E), and then the sections were examined using a light microscope on the basis of the method of Yang et al.[19].

2.6. Measurement of specific antibodies and mouse mast cell protease-1

Whole blood from each mouse was collected, followed by separating serum for Enzyme-Linked Immunosorbent Assay (ELISA). A standard ELISA protocol was used to measure the levels of OVT-specific murine IgE, IgG and IgG1 as previously described with slight variations [20]. Briefly, 96-well microtiter plates were coated either with Apo-OVT or Holo-OVT (2 µg/ well). Sera samples were diluted to 1:2 for IgE, 1:50 for IgG and 1:25 for IgG1. HRP-conjugated goat anti-mouse IgE, IgG and IgG1 antibodies (diluted to 1: 5,000) were applied. The results were reported as optical density (OD). Mouse mast cell protease-1 (mMCP-1) was detected by a commercial ELISA kit according to manufacturer's instructions (eBioscience, USA).

2.7. Cell culture and cytokine analysis

Murine splenocyte culture was performed as described previously [21]. In brief, individual spleens from each mouse were collected aseptically and single-cell suspensions were prepared. Cells (1×10^6 /well) were cultured in a flat-bottom 96-well plate without any stimulation or in the presence of either Apo-OVT or Holo-OVT (50 μ g or 250 μ g/ well) for 72 h (at 37°C in a 5% CO₂ incubator). Supernatants were collected and stored at -20°C until analyses. IL-4, IL-5, IL-13, IL-17E and IFN- γ were determined by commercial ELISA kits (eBioscience, USA), and cell proliferation was measured by WST-1 kits (Cayman Chemical, USA).

2.8. Protein uptake by dendritic cells

Dendritic cells (DCs) were generated from bone marrow cells according to a published work [22-23] with some modification. In brief, bone marrow cells from BALB/c were isolated and cultured in Roswell Park Memorial Institute (RPMI) 1640 complete medium (10% Fetal Bovine Serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin) supplemented with 200 U/mL granulocyte-macrophage colony stimulating factor recombinant murine granulocyte-macrophage colony stimulating factor (rmGM-CSF), recombinant murine IL-4 (rmIL-4) and 50 μ mol/L 2-mercaptoethanol. At day 0, bone marrow cells were seeded at 1.5×10^6 per 90 mm dish in 10 mL RPMI 1640 complete medium containing 200 U/mL rmGM-CSF, 200 U/mL rmIL-4 and 50 μ mol/L 2-mercaptoethanol. At

day 3, another 10 mL RPMI 1640 complete medium containing 200 U/mL of rmGM-CSF were added to the plates. At days 6, half of the culture supernatant was collected and centrifuged, followed by re-suspending the cells in 10 mL of fresh RPMI 1640 complete medium containing 200 U/mL of rmGM-CSF, and it was returned into the original plate. At day 8, all the non-adherent cells were collected and used for the following experiment.

The collected DCs at day 8 were seeded into 24 well plates (1×10^6 /well in 1 mL) in the presence of either fluorescein isothiocyanate (FITC) labeled Apo-OVT or Holo-OVT (10 μ g/well) [24]. The DCs were incubated for 0h, 1h, 2h and 4h, followed by collecting and staining for CD11c. Percentage of CD11c⁺FITC⁺ was measured using FACSCalibur flow cytometer (BD Biosciences, USA).

2.9. Statistical analyses

Data are presented as means $\pm$ standard deviation (SD) and analyzed using Origin 9.0, while significant differences were evaluated by one-way ANOVA and Tukey's post hoc test. The power analysis and sample size were performed using PS: Power and Sample Size Calculation software according to Dupont and Plummer's method [25]. Differences were considered significant if p-value was less than 0.05.

3. Results

3.1. Identification of ovotransferrin loading iron by urea gel electrophoresis

To determine if Holo-OVT were fully loaded with iron, electrophoresis on urea gel was carried out. As Williams et al. had mentioned that the more iron contents chelated on OVT, the faster electrophoretic velocity, and Apo-OVT migrated the slowest on the urea gel [8]. As indicated in Fig.2, almost all Apo-OVT were chelated with one or two Fe^{3+} , and the purity of diferric OVT (Holo-OVT) was 68% according to the Quality One software.

3.2. Assessment of systemic anaphylactic symptoms

Four weeks after initial sensitization, the mice were challenged orally. No evident systemic anaphylactic symptoms were observed in the mice, but histological examination of the small intestines revealed that Apo-OVT and Holo-OVT induced pathological changes (Fig.3). We examined the intestinal structure of each mouse, and only the typical result in every group was chosen in Fig.3. In the control group, villi structure of the small intestine was tidiness and intact (Fig.3A). In the Apo-OVT group (Fig.3B), villi structure of the small intestine was destroyed. Most of villi were atrophied, broken and shed, some may disappear and the laminae propria were exposed. While in the Holo-OVT group, villi structure of the small intestine has just a slight swelling, and in some area, sloughing of enterocytes at the tops of the villi (Fig.3C). Therefore, the structural damage was worse in Apo-OVT group than in

Holo-OVT group.

3.3. Holo-OVT suppressed the generation of specific IgG and IgG1

The immunogenicity and allergenicity of OVT were tested *in vivo* in a mouse model, and the changes between the Apo-OVT group and the Holo-OVT group were well defined. Since the serum from each mouse was not enough for all the ELISA detections, the levels of specific antibodies except mMCP-1 were detected using the mixed sera in each group. From Fig.4 we can see that, serum levels of specific antibodies and mMCP-1 in the Holo-OVT group showed no significant difference with those in control group ($p > 0.05$). However, mice sensitized by Holo-OVT generated significantly lower sIgG and sIgG1 levels than that in the Apo-OVT group ($p < 0.001$, Fig. 4B and 4C). Serum levels of mMCP-1 and sIgE showed no significant difference between the Apo-OVT group and the Holo-OVT group ($p > 0.05$). However, the higher levels of sIgE from serum was observed in the control group when compared with the mice sensitized by Apo-OVT and Holo-OVT ($p < 0.01$, Fig. 4A).

3.4. Alteration of T-helper type 1/type 2 cytokine ratios

In this study, we detected IL-4, IL-5 and IL-13 as Th2 cytokines, IFN- γ as Th1 cytokine, and IL-17E as Th17 cytokine. Levels of IL-13 and IFN- γ were elevated in Apo-OVT group compared with the control group (Fig.5A and 5C), and the increase in the level of IL-13 was dose dependent ($p < 0.01$). Holo-OVT led to decrease in IL-13, IL-17E and IFN- γ production

in splenocyte cultures ($p < 0.05$, Fig. 5A, 5B and 5C). IL-4 and IL-5 were not detectable (according to the ELISA kit sensibility) in all the three groups. Significant differences existed in the splenocyte proliferation between Apo-OVT group and Holo-OVT group ($p < 0.001$, Fig. 5D).

3.5. Uptake patterns by DCs between Apo-OVT and Holo-OVT

To better understand the difference of potential allergenicity between Apo-OVT and Holo-OVT, antigen uptake behavior was monitored by DCs *in vitro*. The DCs were incubated with FITC labeled Apo-OVT or Holo-OVT for 0h, 1h, 2h and 4h. Uptake of FITC labeled Apo-OVT and Holo-OVT by DCs was determined by flow cytometry. With the extension of incubation time, the number of antigen uptake was increased, and the uptake of FITC-Holo-OVT was higher than that of Apo-OVT ($p < 0.01$, Fig. 6).

4. Discussion

Although a lot of proteins can be chelated with iron, not much information is available about the health risks of these new protein derivatives. Since the iron-chelated proteins have different molecular structure from the iron-free ones [12-15], it is necessary to assess the allergenicity of these novel protein derivatives. Previous studies have demonstrated that iron-loaded Bet v 1 and Bos d 5 could inhibit immune response [12-13]. It is not clear if iron-chelated OVT has similar lower allergenicity as with other iron-chelated proteins.

Therefore, the allergenicity of Apo-OVT and Holo-OVT were assessed and compared in this study, and the results demonstrated that iron-induced chelation could alleviate the potential allergenicity of OVT in the BALB/c mouse model.

Food allergy is Th2-dominant allergic immune responses including systemic, gastrointestinal, and mucosal immune systems symptoms [26-29]. In this study, BALB/c mice were sensitized with Apo-OVT and Holo-OVT, respectively. The obvious systemic anaphylactic symptom was not observed in OVT-challenged mice, but intestinal allergic symptom was evidenced through histological examination. When the levels of antigen-specific antibodies were detected, Apo-OVT-specific IgG and IgG1 were significantly increased compared with the control group. However, Apo-OVT-specific IgE was very low, even lower than that in control group. This suggested that the pathway of systemic anaphylaxis in the mouse model caused by OVT was an alternative pathway rather than the classic pathway [30]. Several researches had indicated that the quantity of antigen-specific IgG antibody produced during the course of an immune response could inhibit IgE-mediated anaphylaxis [31-32], and IgG subclasses determined pathways of anaphylaxis in mice [33]. IgG1 is one of four IgG subclasses that exist in mice [33], and is also a Th2-related immunoglobulin like IgE [28]. IgG1 mediated anaphylaxis was triggered by basophiles through an alternative pathway of systemic anaphylaxis, while IgE mediated anaphylaxis was triggered by mast cell in the classic pathway [30, 33-34]. Therefore, the allergic response in the mouse model caused by OVT was induced by IgG1 through the alternative pathway. This could also be reflected by the results of mMCP-1 levels. mMCP-1 is an indicator of the classic pathway [30], because the

allergic response in the mouse model caused by OVT was induced by IgG1 through the alternative pathway, release of mMCP-1 during mast cell degranulation in the Apo-OVT group and the Holo-OVT group were lower than that in the control group.

The allergenicity of OVT was also assessed by detecting the cytokines (IL-4, IL-5, IL-13, IL-17E and IFN- γ) from splenocyte *in vitro*. Here, IL-4, IL-5 and IL-13 are Th2-related cytokines, IFN- γ is Th1-related cytokine, and IL-17E is Th17-related cytokine. IL-17E was detected because Gu et al reported that it could enhance Th2 responses [35], however, the levels of IL-17E in Apo-OVT group were much lower than that in control group. This may be explained by the different mechanism of developing OVT allergy in BALB/c mice. In the present study, IL-4 and IL-5 were not detectable (according to the ELISA kit sensibility) in the sera of all the groups, while levels of IL-13 were elevated compared with the control group. In food allergy, Th2 cytokines are considered to be a key factor for developing an allergy [27]. However, interestingly, levels of IFN- γ were also remarkably high in Apo-OVT group compared to control group. The up-regulation of IFN- γ in Apo-OVT treated mice could be a result of severe allergic responses in mice, which needed to secrete more Th1 cytokine to keep the balance of Th1/Th2 ratio, similar allergic responses were found in milk allergy reported earlier [24]. Morafo [36] and van Wijk [37] had pointed out that an antigen-specific mixed Th1 and Th2-type cytokine response was very common in food allergy. Accordingly, our results showed that both Th1 and Th2 phenomena were involved in the development of OVT allergy in the BALB/c mouse model.

As a whole, OVT induced allergic reaction in mice was mediated by IgG1 through an alternative pathway, and both Th1 and Th2 immune response were involved in mice. When OVT was chelated with iron (Holo-OVT), the structure was different from natural iron-free OVT (Apo-OVT) [14-15]. There is a growing body of evidence that the allergenicity of food allergens were closely linked to their structure [38-41]. In this study, iron chelation greatly reduced the allergenicity of OVT, which was supported by the significantly decreased levels of OVT-specific IgG and IgG1, as well as the Th-1 cytokine IFN- γ and Th2-type cytokine IL-13 in Holo-OVT sensitized mice compared to Apo-OVT group ($p < 0.05$). In addition, the levels of OVT-specific antibodies and Th-1 and Th2-type cytokines showed no significant difference between Holo-OVT group and control group ($p > 0.05$). Moreover, the actual gastrointestinal hypersensitivity of mice was measured by intestinal pathological examination, and Holo-OVT led to a weak immune reaction compared with Apo-OVT. All the results indicated that iron-chelated OVT was less allergenic than Apo-OVT, and iron attenuated immune responses through maintaining Th1/Th2 immune balance in the mouse model.

To further compare the allergenicity of Apo-OVT and Holo-OVT, we inspected protein interactions with DCs *in vitro*. Generally, it is considered that the uptake, processing and presentation of antigens on the surface of antigen presenting cells such as DCs determines the type and severity of immune response [42]. However, in this study, uptake of Holo-OVT by DCs occurred at a much higher rate than that of Apo-OVT, and it seemed to get an opposite result compared to the allergic capacity tested *in vivo*. Actually, our results were similar to Stojadinovic et al [24]. They reported that uptake of BLG by DCs was reduced through

cross-linking while its immune-stimulating capacity was improved. It reminded us that it was too simple to define the relationship between DCs and the allergenicity of allergens only using antigen uptake by DCs. The lower allergenic capacity (as measured *in vivo*) but higher uptake by DCs of Holo-OVT suggested that intestinal transport and processing of this protein derivative might be altered, which may be related to the different structure between Apo-OVT and Holo-OVT.

There are some limitations of this study. Although Holo-OVT was produced from Apo-OVT, the purity of Holo-OVT was not as high as Apo-OVT. There are three forms of OVT, which are Apo-OVT, Mono-OVT and Holo-OVT, according to the number of chelated iron atoms per protein molecule [9]. In this study, as indicated in urea gel electrophoresis, almost all Apo-OVT were chelated with one or two Fe^{3+} , but the purity of di-ferric OVT (Holo-OVT) was only 68% according to the Quality One software. There was still Mono-OVT in Holo-OVT. Therefore, it is necessary to further validate how iron-chelation reduces the allergenicity of OVT using higher purity of Holo-OVT. In addition, it was too simple to define the relationship between DCs and the allergenicity of allergens only using antigen uptake by DCs as mentioned above. The digestibility of OVT and transport in the gastrointestinal tract as well as the processing and presentation by DCs should be taken into account in the future study.

Our finding confirmed our hypothesis that iron-chelation could decrease the allergenicity of OVT *in vivo*, which implied that in the future, to produce iron-chelated OVT (Holo-OVT) or

hypoallergenic eggs containing Holo-OVT may be effective to improve the efficacy of allergen immunotherapy against egg allergy. OVT in egg white is synthesized in the hen oviduct. Expression of the transferrin gene in the oviduct can be regulated by levels of oestrogen and progesterone [43], and this expression may be affected by iron levels [44]. Although OVT in egg white is normally without ferric ions [6], by controlling the hens' diet, it may be possible to produce a hypoallergenic egg containing Holo-OVT. In addition, it was reported that iron (Fe^{3+}) can be easily chelated to OVT at $\text{pH} > 7.0$ [45]. When we use OVT as an ingredient into the food matrix, it may also be beneficial to produce Holo-OVT by adding Fe^{3+} solution and adjusting the pH of the food matrix.

In summary, it was the first time the allergenicity of ovotransferrin was assessed in a BALB/c mouse model, and we provide evidence that the allergic response in the mouse model caused by OVT was IgG1-mediated anaphylaxis induced through the alternative pathway, and both Th1 and Th2 immune response were involved in mice. Holo-OVT elicited weaker allergic response in mice than Apo-OVT, and iron chelation attenuated immune responses through maintaining Th1/Th2 immune balance in the mouse model.

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Figure legend

Fig.1. Outline of the mouse model design. Mice were sensitized to 1 mg of Apo-OVT or Holo-OVT and 10 μ g of cholera toxin for 4 weeks, twice per week, followed by oral challenge with 20 mg of Apo-OVT or Holo-OVT. The control group received distilled water instead of protein. Clinical signs of allergy were recorded immediately after oral challenge and 40 min later, all mice were euthanized for the collection of blood and spleen. CT, cholera toxin; Apo-OVT, iron-free ovotransferrin; Holo-OVT, iron-chelated ovotransferrin. A, Apo-OVT group; H, Holo-OVT group; C, CT group as control.

Fig.2. Identification of Apo-OVT and Holo-OVT by urea gel electrophoresis. The urea gel was made up by 1.4 mL 30% Acryl amide-0.8% Bis Solution (37.5:1), 2.1 g urea, 0.7 mL 8 $\times$ TBE, 5 μ L N,N,N',N'-yteramethyl ethylenediamine, and 25 μ L 10% Ammonium persulfate followed by adding H₂O to 7 mL. Apo-OVT and Holo-OVT were diluted in sample buffer containing 10% (v/v) 8 $\times$ TBE, 6 mol/L urea, 50% (w/v) sucrose and 0.2% (w/v) bromophenol blue. The parameters for urea gel electrophoresis were carried out at 150V for 4 h at 4°C.

Fig.3. Typical histological alterations in the intestine of the orally challenged mouse. Jejunum samples were collected and fixed in neutral-buffered formaldehyde and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin, and then the sections were examined using a light microscope. A, jejunum of control group; B, jejunum of Apo-OVT group; C, jejunum of Holo-OVT group

Fig.4. OVT-specific IgE (A) , IgG (B) , IgG1 (C) and mMCP-1 (D) in sera after the oral challenge. OVT-specific IgE, IgG and IgG1 activity was measured by ELISA in triplicates for each group. HRP-conjugated goat anti-mouse IgE, IgG and IgG1 antibodies were applied. The results were reported as optical density (OD). The concentration of mMCP-1 in each mouse was detected by a commercial ELISA kit according to manufacturer's instructions. Results were presented as the means $\pm$ SD. Different letters above the bars represented significant differences ($p < 0.05$).

Fig.5. Cytokines producing and cell proliferation of the splenocyte stimulated by Apo-OVT or Holo-OVT: levels of IL-13 (A), IL-17E (B), IFN- γ (C) and cell proliferation (D). The spleen was collected from each mouse, and splenocytes were isolated and cultured at 1×10^6 /well in triplicate wells as unstimulated (control) or stimulated with Apo-OVT or Holo-OVT (two doses were set, which were 50 μ g/well and 250 μ g/well) for 72 h. The cytokine concentration of cell culture supernatants for IL-4, IL-5, IL-13 (A), IL-17E (B), and IFN- γ (C) was determined by ELISA. Cell proliferation was measured by WST-1 kits according to manufacturer's instructions. Results were presented as the means $\pm$ SD. Different letters above the bars represented significant differences ($p < 0.05$).

Fig.6. Uptake behavior of FITC labeled Apo-OVT and Holo-OVT by DCs tested by flow cytometer. The DCs were seeded into 24 well plates (1×10^6 /well in 1 mL) in the presence of

either fluorescein isothiocyanate (FITC) labeled Apo-OVT or Holo-OVT (10 μ g/well) and incubated for 0h, 1h, 2h and 4h, followed by collecting and staining for CD11c. Percentage of CD11c⁺FITC⁺ was measured using flow cytometer. Results were presented as the means $\pm$ SD. Different letters above the bars represented significant differences ($p < 0.05$).

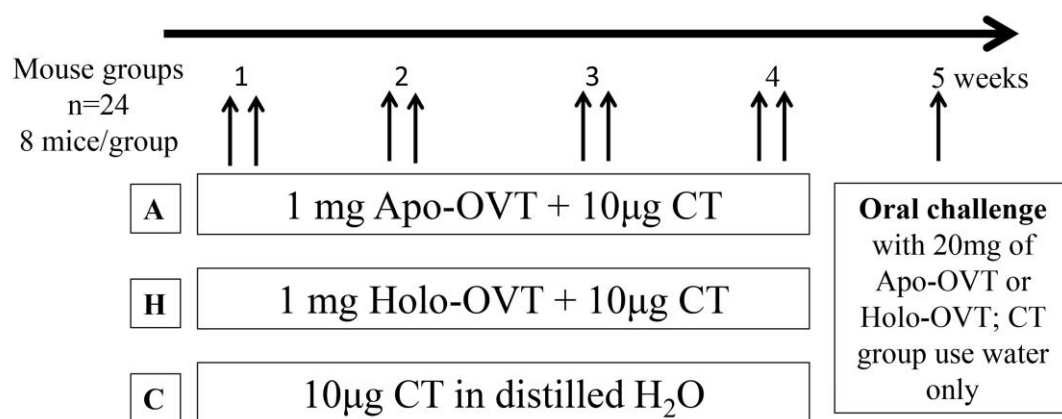


Figure 1

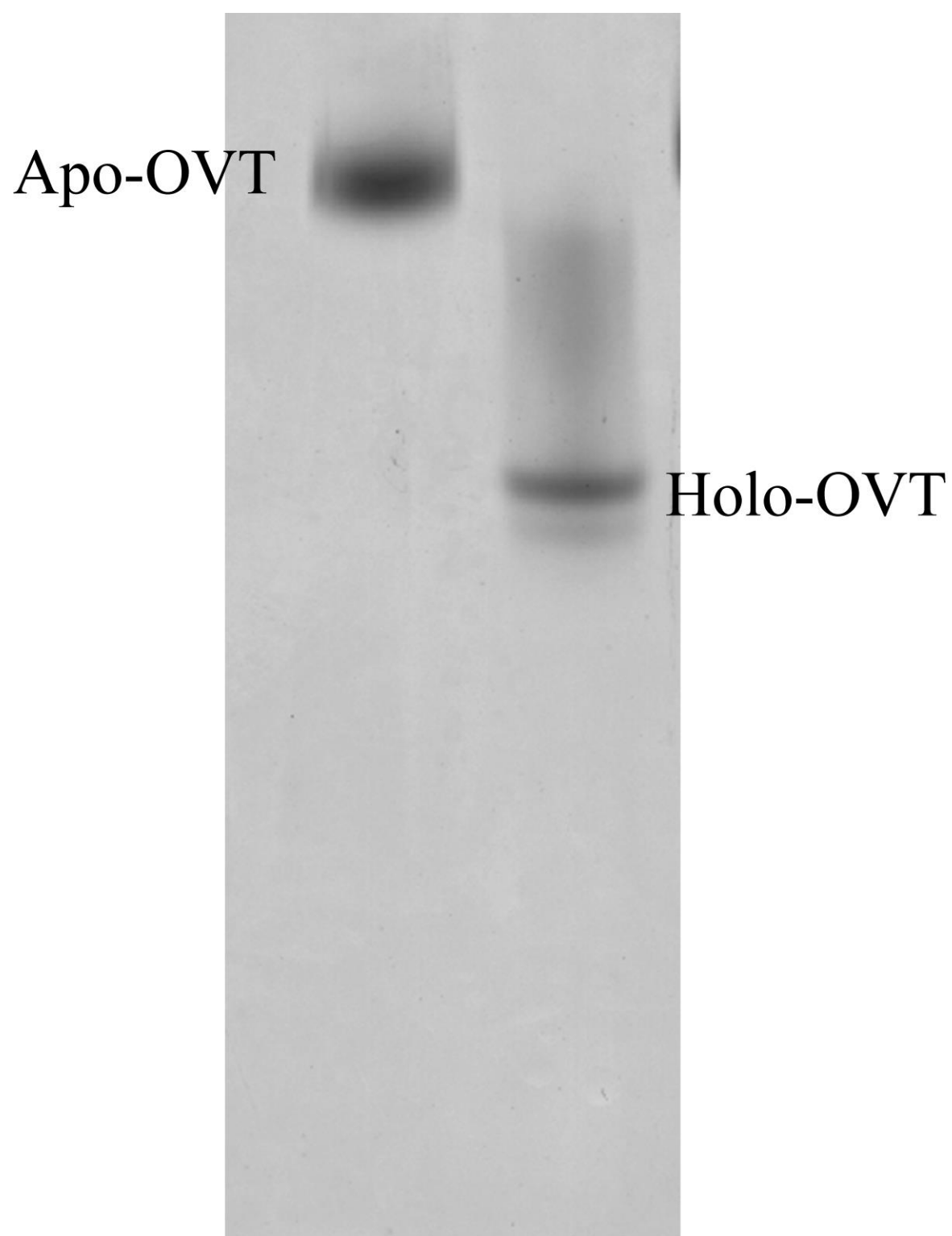


Figure 2

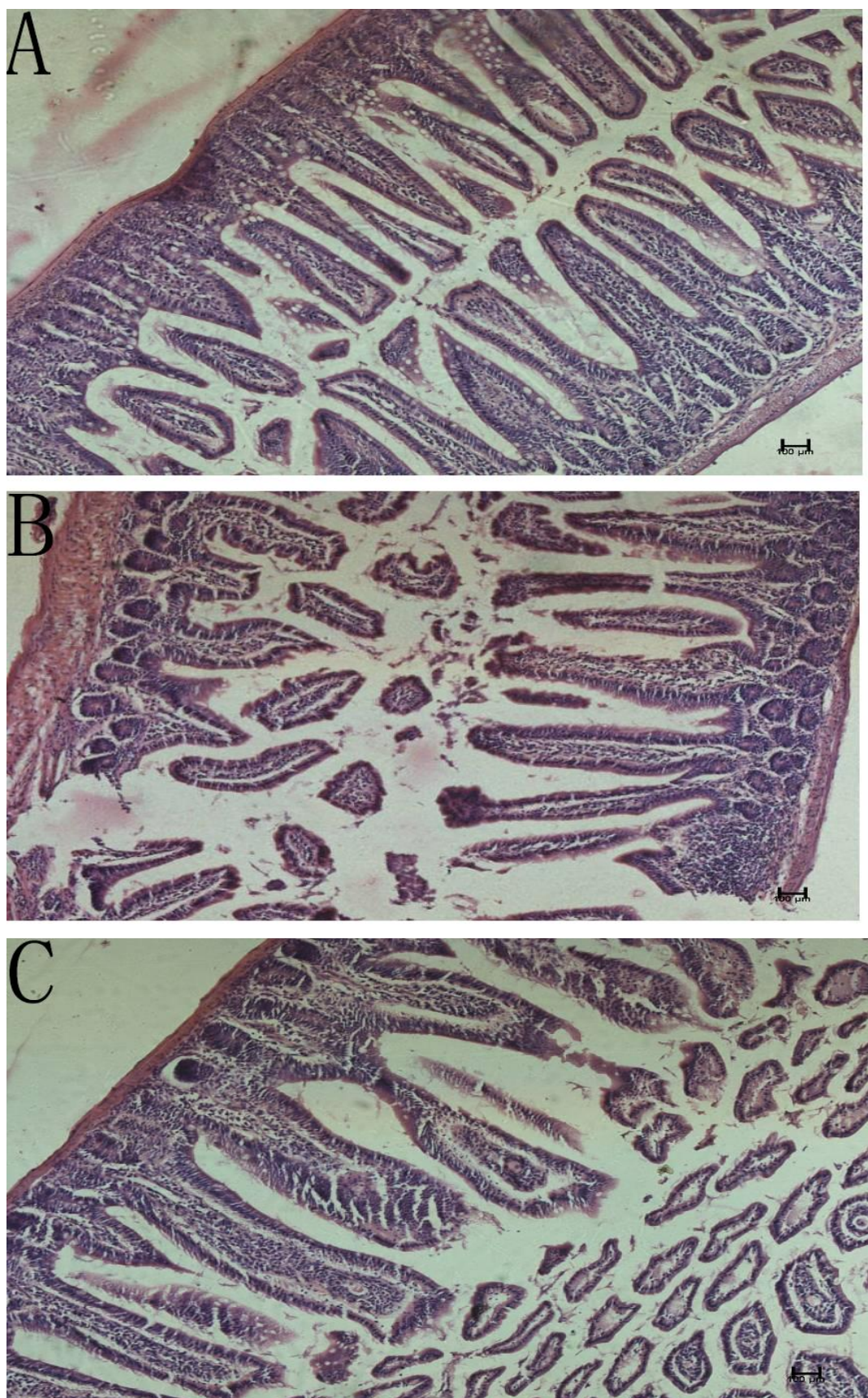


Figure 3

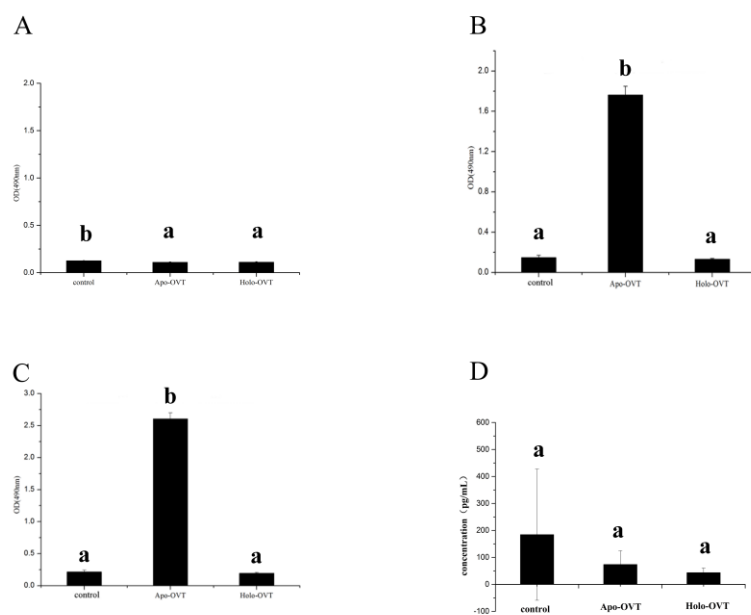


Figure 4

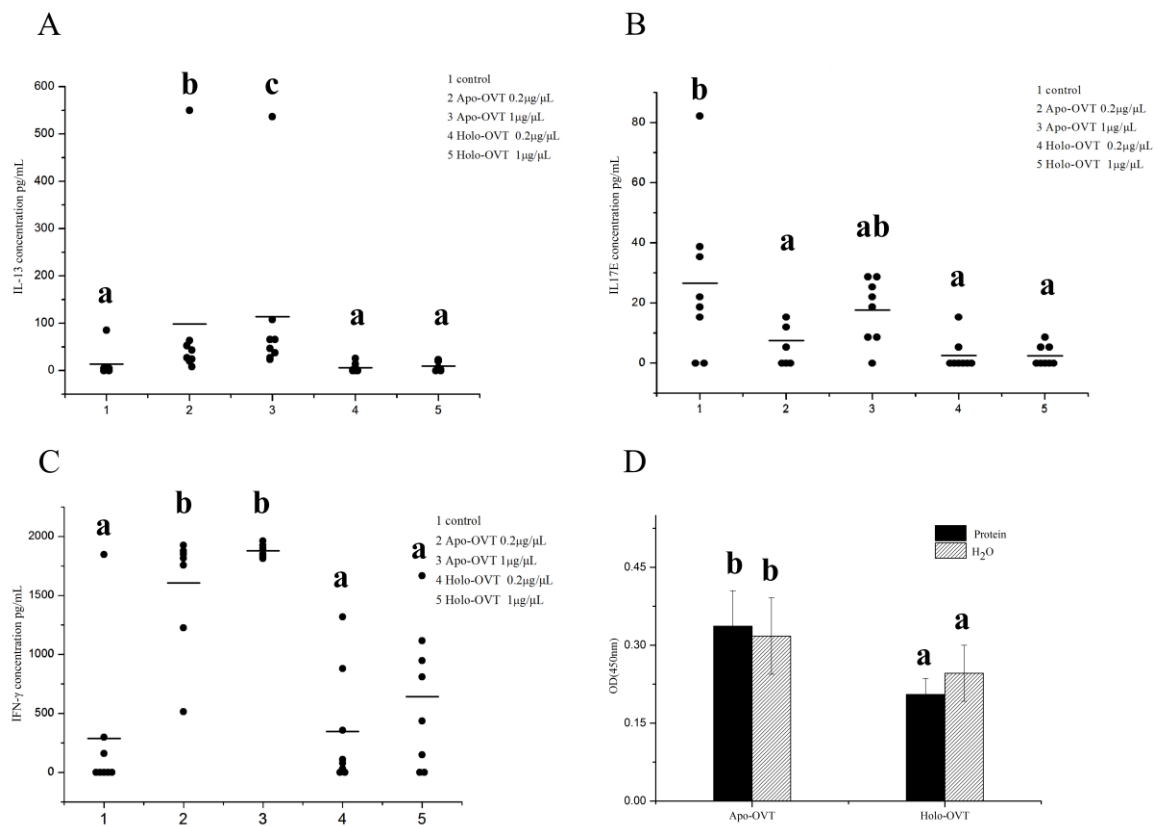


Figure 5

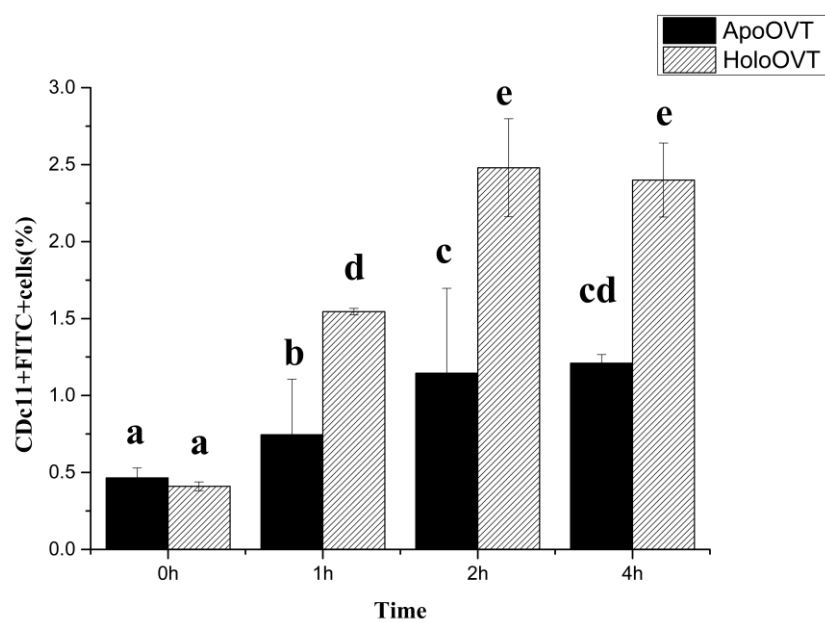


Figure 6