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Full Length Research Paper

Modeling a murine model of immunoglobulin-E (IgE)-mediated qingkailing injection anaphylaxis

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Qingkailing injection (QKLI) is a classic compound traditional Chinese medical injection (TCMI). It can cause anaphylatic shock and death in clinic. Modeling a murine model of qingkailing injection anaphylaxis appears to be essential. In this study, for the first time, we used several strategies to prepare cationic bovine serum albumin (cBSA) and QKLI-cBSA conjugate. The native bovine serum albumin (nBSA), cBSA and QKLI-cBSA conjugate were separated by cationic exchange method and also identified by ultraviolet-visible (UV-Vis) and fourier transform infrared (FT-IR) spectrometry. Balb/c mice were then treated with subcutaneous injection of QKLI-cBSA plus alum. Serum was analyzed for total IgE and histamine levels by enzyme-linked immunosorbent assays (ELISAs), respectively. Lung reactions were evaluated by analyzing lung pathologic changes. QKLI-cBSA group mice were significantly and successfully sensitized. QKLI-cBSA conjugate significantly increased total IgE and Histamine in Balb/c mice serum. Moreover, histological examination of QKLI-cBSA conjugate treated mice showed acute injury with pulmonary alveoli and injury of trachea, as well as neutrophilic cell infiltration. In conclusion, we successfully established a murine model of immunoglobulin-E (IgE)-mediated qingkailing injection anaphylaxis. This model should provide a useful tool for detecting allergens in QKLI and for exploring new allergens-detecting approaches of other TCMIs.

Key words: Qingkailing injection, murine, immunoglobulin-E (IgE)-mediated anaphylaxis, Qingkailing injection (QKLI) - cationic bovine serum albumin (cBSA) conjugate.

INTRODUCTION

Qingkailing injection (QKLI) is a modified formulation in a new dosage from a well-known classic formulation “An Gong Niu Huang Wan”, which contains four plant species (*Fructus gardeniae, Radixisatidis, Radix scutellaria, and Flos lonicerae*), a number of animal products (Beijing College of TCM, 1975). The QKLI is not only cheap, but also can be used extensively by Chinese doctors to cure cerebral ischemia, intracerebral hemorrhage (ICH), neurotoxic damage, bacterial meningitis and vascular dementia (Liu et al., 2006; Hua et al., 2008; Yue et al., 2006; Hu et al., 1992; Tian, 1998). In 1992, QKLI was specified as a "must-have" preparation for TCM emergency clinics by the State Administration of Traditional Chinese Medicine of China (SATCM) (Lee et al., 2000). However, with the wide application of QKLI, the reports of adverse reactions related to QKLI appear to have increased significantly in China over the past ten years. From 2001 to 2007, the Chinese National Adverse Drug Reaction Monitoring Center (CNARMC) received notices of 580 serious adverse drug reaction (ADR) cases caused by QKLI, including 20 (3.45%) fatalities (Huang et al., 2007). Adverse drug reactions (ADRs) reported for QKLI from 2001 through 2007 mainly included anaphylactic shock (Niu and Zhou 2000) (33%), dyspnoea (23%), hypotension (10%), cataphora (5%), pulmonary edema (5%), laryngeal edema (2%), and convulsions (2%). Since the explosion of adverse event, CNARMC posted a bulletin in which it warned of the potential for severe ADRs to QKLI (SFDA, 2009b). Those safety concerns caused a reduced usage of QKLI and increasingly questioned its rationality, and thus ultimately lead to the "crisis of confidence", to the whole traditional Chinese medicine industry. Qingkailing injection, the most common and most complicated of TCMIs, became the focus of governments and scientists throughout China due to the strong potential hypersensibility shown

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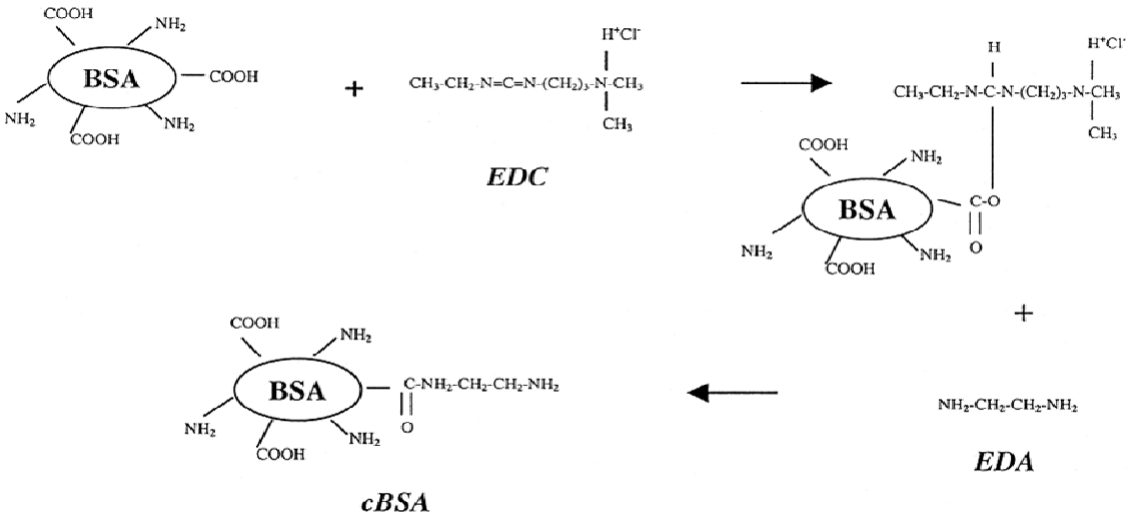

Figure 1. The fluidogram of cBSA preparation.

in clinic applications. Therefore, there are strong economic and safety reasons for establishing methods for detecting potential allergens in QKLI.

Immediate anaphylaxis, generally being considered as the most frequent and severe adverse reaction induced by QKLI. Taking the mechanism of immediate anaphylaxis into consideration, obtaining QKLI-specific antibodies is one of the key steps in establishing methods for detecting allergy. However, there is no time to draw blood from autopathes during salvages. Thus, we have to modeling an anaphylaxis animal model to obtain QKLI-specific antibodies. At present, the anaphylaxis animal models of macromolecule weight compound (MMWC), such as, cow's milk (Xiu et al., 1999), peanut (Xiu et al., 2005) and latex (Ramos et al., 2007), had been widely used for detecting allergens. However, the anaphylaxis animal models of traditional Chinese medicine injection are still scarce. In our previous work (Xu et al., 2010), we found that QKLI include no MMWC but low weight molecule compound (LWMC, 1.0-1.5 KD). Nevertheless, LMWC cannot directly induce immunological response, unless coupled with carrier protein (Ilkka and Seppälä, 2004). As it has no MMWC, the QKLI has significant differences from conventional MMWC in establishing anaphylaxis animal models. Obviously, establishing an animal anaphylaxis model for QKLI will be a challenging work.

In the current study, for the first time, we used several strategies to prepare QKLI-cBSA conjugate based on a Mannich-type method, in which carrier protein (native BSA, nBSA) is cationized by ethylendiamine, then the QKLI-cBSA conjugate is prepared by condensing the ethylendiamine group in the cationic protein (cationic BSA, cBSA) with formaldehyde and the -hydrogen adjacent carbonyl in QKLI. The cBSA and QKLI-cBSA conjugate were also firstly separated and purified by cationic ion exchange methods. After purification, the cBSA and QKLI-cBSA conjugate were then identified by FT-IR spectrometry. BALB/c mice were then treated with subcutaneous injections of QKLI-cBSA plus alum. Serum was analyzed for total IgE and histamine levels by ELISAs. Lung reactions were evaluated by analyzing lung pathologic changes.

MATERIALS AND METHODS

Materials

Qingkailing injection was purchased from HeBei ShineWay Pharmaceutical Company (ShiJiazhuang, China) . 1-Ethyl-3-[3-dimethylaminopropyl]- carbodiimide hydrochloride (EDC), 37% formaldehyde, ovalbumin (OVA), 2-(N- morpholino)-ethane sulfonic acid (MES) and alum were obtained from Pierce (Rockford, IL, USA). Native bovine serum albumin (nBSA) was purchased from Merck (Darmstadt, Germany). Mouse IgE ELISA KIT was obtained from BioLegend (San Diego, CA, USA) . Mouse Histamine ELISA KIT was obtained from Rapid Bio Lab (California, USA). Ethylenediamine (EDA), common reagents and other organic solvents were purchased from Guangzhou Chemical Reagent Company (Guangzhou, China).

Animals

Female, 6-week-old, BALB/c mice were purchased from The Laboratory Animal Research Center of the Southern Medical University (Guangzhou, China). The animals used for the experiment were treated according to protocols evaluated and approved by the ethical committee of Southern Medical University.

Preparation of cBSA

The fluidogram of cBSA synthesis (Jean et al., 1999) is shown in Figure 1. Briefly, 200 mg of crystallized native bovine serum albumin (nBSA) was dissolved in 2 ml of conjugation buffer (0.1 M MES pH 4.7). Anhydrous ethylenediamine (EDA) solution was pre-

pared by mixing 2.68 ml of EDA and 20 ml of conjugation buffer in an ice bath; the pH value was readjusted to 4.7 with 6 N HCl and the solution cooled to room temperature (RT). This EDA solution was added slowly to the nBSA solution followed by 150 mg of 1-ethyl-3-[(3-dimethylaminopropyl) -carbodiimide hydrochloride] (EDC) and incubated at RT for 4 h with continuous magnetic stirring.

The reaction solution was then added into 20 ml cationic ion-exchange column (BioLogic DUO FLOW system, Bio-Rad, USA)which was filled with SP sepharose fast flow base. The separation for cBSA was described (Chen et al., 2004) previously. After loading samples, the column was washed with 100 ml of pH 6.8 phosphate buffer (10 mM) to remove the non-reacted nBSA. Subsequently, the cationic BSA (cBSA) was eluted with 100 ml of pH 10.6 phosphate buffer (10 mM) and collected by BioLogic Fraction Collector (Bio-Rad, USA). The collections were monitored by UV-Vis spectrometry. The fractions which showed highest absorbance were pooled and concentrated to 4 ml by using JumboSep ^{TM} centrifugal device (Pall, Mexico). Then the concentrated cBSA were dialyzed exhaustively against deionized water for 72 h. Finally, the salt-free cBSA was lyophilized, identified by UV-Vis and FT-IR spectrometry and stored at -20°C.

Preparation of QKLI-cBSA conjugate for immunization

The preparation of QKLI-cBSA conjugate was based on the Mannich reaction (Gerg, 1996). Briefly, 4 mg of QKLI freeze-dried powder was dissolved into 0.4 ml of deionized water. Then 2 mg of cBSA was dissolved in 0.2 ml of conjugation buffer (0.1 M MES pH 4.7). After dropwise addition of QKLI solution into the cBSA solution, the mixture was stirred gently, then 50 l of conjugation reagent (37% formaldehyde) were added into the mixture and immediately incubated for 24 h at 37°C.

The reaction solution was then added into 20 ml cationic ion-exchange column which was filled with SP sepharose fast flow base. Subsequently, the conjugate was eluted with buffer A (10 mM PBS, pH = 7.2), and the non-conjugated cBSA was eluted by buffer B (10 mM PBS, 0.5 M NaCl, pH = 7.2). All collections were collected by BioLogic Fraction Collector, respectively. The collections were monitored by UV- Vis spectrometry. The fractions which showed highest absorbance were pooled and concentrated to 4 ml by using JumboSep ^{TM} centrifugal device. Then the concentrated products were dialyzed exhaustively against deionized water for 72 h. Finally, the salt-free conjugate was lyophilized, identified by UV-Vis and FT-IR spectrometry and stored at -20°C.

Identification of cBSA and QKLI-cBSA conjugate

Ultraviolet-visible (UV-Vis) analysis of nBSA, cBSA, QKLI-cBSA conjugates

The nBSA, cBSA and QKLI-cBSA conjugate were analyzed spectrometically in a quartz cuvette with a 1- cm light path, employing a double bundled UV-Vis spectrometer, mode Lambda 35 (PerkinElmer, USA). Absorption spectra of all samples in PBS were scanned from 200 to 400 nm at 1-nm intervals. The scanning speed was 12 nm·s^{-1} and the bandwidth was 1 nm.

Investigation of coupling by Fourier-transform infrared spectrometry

FT-IR spectra of nBSA, cBSA and QKLI–cBSA conjugate were recorded in the region of 400 to 3500 cm^{-1} at room temperature on a FT-IR spectrometer, model Avatar 380 (Thermo Nicolet, USA). The spectral resolution was set at 4 cm^{-1} and 32 scans; the test sample was dispersed into a potassium bromide pellet and the Thermo Electron software, OMNIC 6.0, was used to analyze the results.

Animal immunization and serum collection

Groups of 30 6-week-old male Balb/c mice if weight 18 to 22 g received subcutaneous injection of 50 g of QKLI-cBSA, OVA, QKLI, cBSA or NS in 0.15 ml of sterile saline plus 0.15 ml of aluminum hydroxide as adjuvant, respectively. Booster injections were carried out on the 14th, 21st, 28th and 35th day after the primary doses. Mice were bled from the retro-orbital plexus just before first immunization (day 0) and at day 14, 21, 28 and 35 days after first injection. Posterior blood collections were performed 2 min after their skin was scratched. After blood collection, serums were allowed to repose for 30 min at RT and thus slightly centrifuged in a bench centrifuge for 10 min at RT, the clean samples finally stored at -20°C until use.

Determination of total IgE level in mice serum

The IgE ELISA kit from BioLegend, Inc. was used to determine the total IgE content in mice serum. The assay was performed according to the protocol provided by the supplier. Briefly, ELISA plates (96-well EIA/RIA plate, 96- well easy wash ^{TM}, high binding, Corning, New York) were coated with capture antibody diluted in carbonate buffer (0.05 M, pH 9.6) and incubated at 4°C, over night. Unbound extract was discarded and the plates were blocked with dilution buffer (0.1% BSA in PBS) at 37°C. After washing (0.05% Tween-20 in PBS), IgE standards and serum samples were added. Following incubation, plates were washed and biotin-labeled anti-mouse IgE antibody added. After incubation, plates were washed and streptavidin alkaline phosphatase conjugate added. Subsequently, plates were washed and TMB substrate added. Reactions were allowed to develop at room temperature in the dark and stopped by 2 N H_2SO_4 solution. Finally, the absorbances were measured in a micro-plate reader (Model 580, Bio-Rad, USA) at 450 nm.

Determination of histamine level in mice serum

The histamine ELISA kit from Rapid. Bio. Laboratories, Inc. was used to determine the histamine content in mice serum. The assay was performed according to the protocol provided by the supplier. Briefly, histamine standards and serum samples were added to ELISA plates which had been pre-coated with anti-mouse histamine antibody. Following incubation, plates were washed and biotin-labeled anti-mouse histamine antibody added. After incubation, plates were washed and streptavidin alkaline phosphatase conjugate added. Subsequently, plates were washed and added. Reactions were allowed to develop at room temperature in the dark and stopped by adding 2 N H_2SO_4 solution. Finally, the absorbance was measured in a micro-plate reader at 450 nm.

Histological examination

For assessment of pathologic alterations, dissected lung tissues were washed with normal saline (5 ml) and then placed in 10% neutral-buffered formaldehyde for 1 week. After fixation, lung specimens were embedded in paraffin wax, and five-micrometer sections were cut and stained with hematoxylin and eosin dye for morphology. Images of selected sections were captured at X100 magnification using a zoom digital camera (Kodak Company, Rochester, NY, USA). One pathologist blind to the study groups

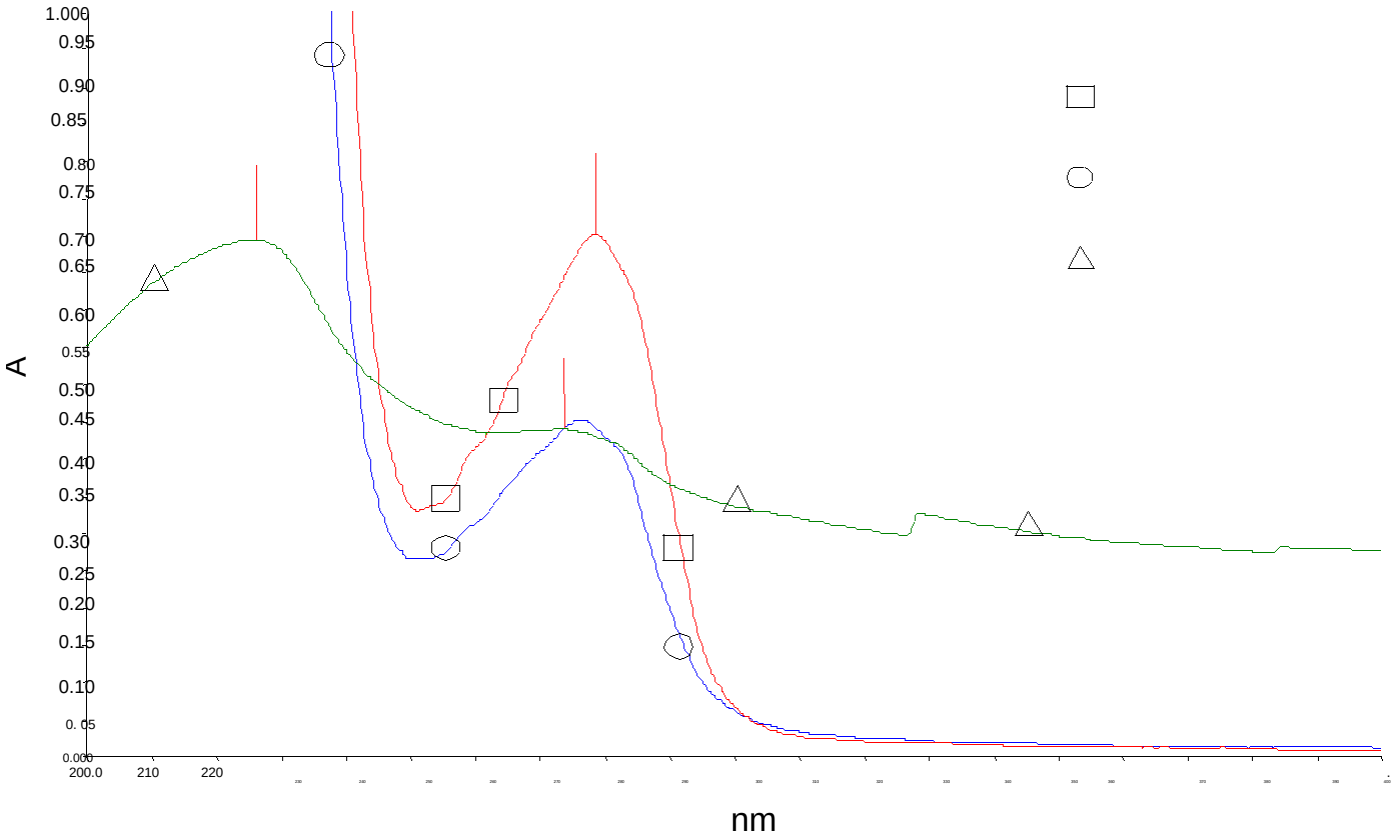

Figure 2. The UV-Vis spectra of nBSA (A), cBSA (B) and QKLI-cBSA conjugate (C) in MES buffer.

performed all the histological examinations.

Statistical analysis

All experiments were repeated at least three times. Experiment data were expressed as mean ± SD and statistical differences between groups were performed by one-way analysis of variance (ANOVA) followed by Student Newman-Keuls test. Differences were considered significant at $P<0.05$. All computations were performed using SPSS 16.0 software.

RESULTS AND DISCUSSION

UV-Vis identification of nBSA, cBSA and QKLI-cBSA conjugate

The UV-Vis absorption spectra of nBSA, cBSA and QKLI-cBSA conjugate are shown in Figure 2, respectively. The maximum absorbing wavelength in cBSA UV–Vis spectra, which are characteristic of protein, were slightly blue-shifted from 278 to 276 nm compared with nBSA. This might be explained in terms of increased cBSA polarity due to induction of excessive cationic groups, amino-ethylamine groups, into nBSA. The spectra of QKLI– cBSA conjugate showed a broad characteristic absorption band around 225 nm and another band around 275 nm, indicating a combination band of 275 and 225 nm due to QKLI and cBSA, respectively.

FT-IR identification of carrier proteins and QKLI-cBSA conjugate

The FT- IR spectra of nBSA, cBSA and QKL-cBSA obtained by dispersing them into a potassium bromide pellet are shown in Figure 3, respectively. The strong bands at 1651 and 1550 cm^{-1} in nBSA (Figure 3A) were attributed to amides I and II. cBSA exhibited almost the same range (Figure 3B) as nBSA, but some intensities were different. On the other hand, the band at 1470 to 1370 cm^{-1} was ascribed to $_s$ (-COO-) in nBSA, but the cBSA and QKLI-cBSA had no signals at this range, indicating the decrease of carboxyl in cBSA. Moreover, a strong band was observed at 1082 cm^{-1} and a broad strong band at 3384 cm^{-1} corresponded to $_s$(R-CH_2-N) and $_s$(NH) in cBSA, respectively. This observation demonstrated the substitution of carboxylic groups by ethylendiamine in cBSA.

QKLI was conjugated in cBSA provided an increase in carbonyl absorption at 1650 and 1560 cm^{-1}, ascribable to $_s$ (C=O) and $_{as}$ (-COO-) in QKLI compared with cBSA. Conjugation of QKLI and cBSA significantly decreased

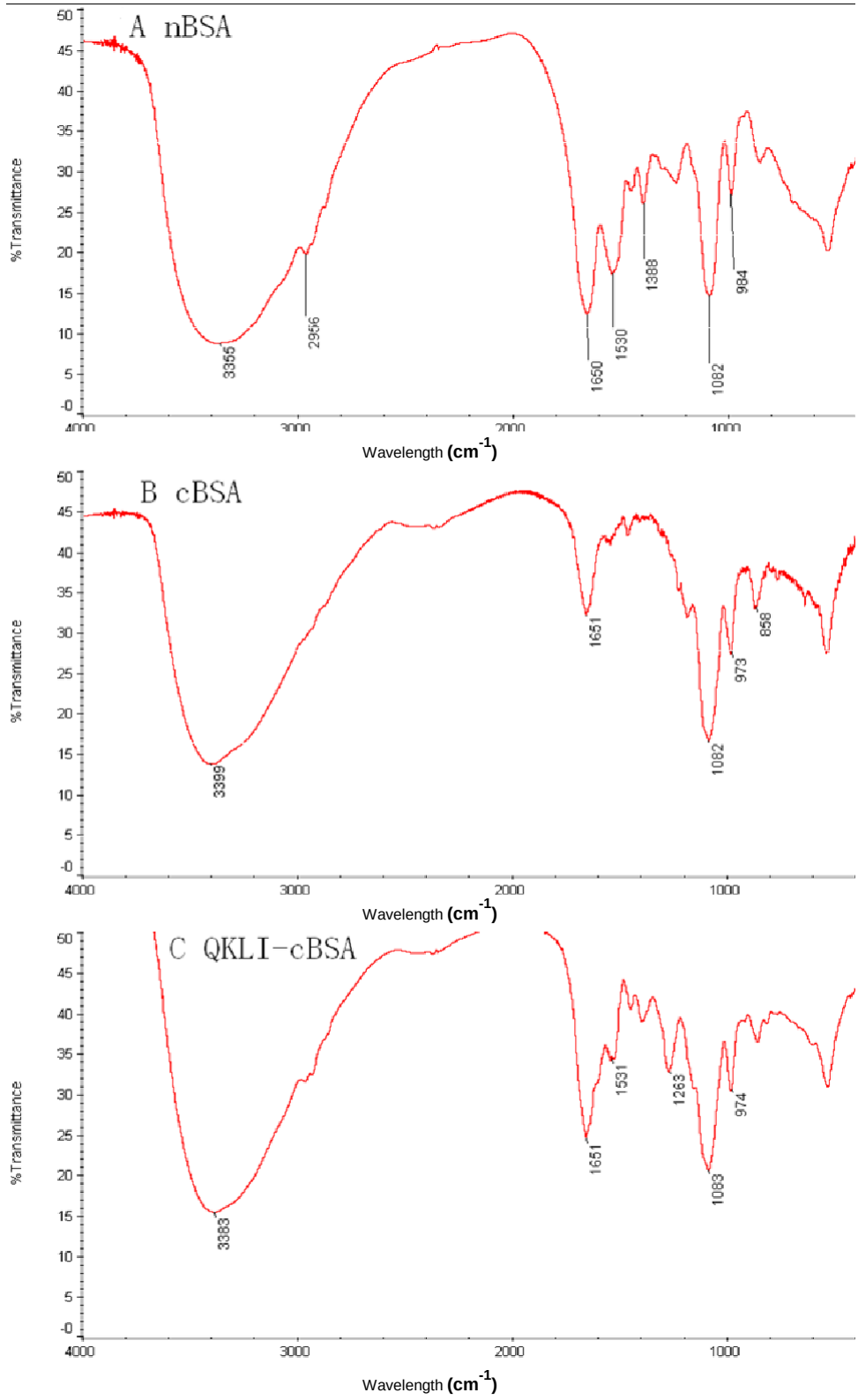

Figure 3. The FT-IR spectra of nBSA (A), cBSA (B) and QKLI-cBSA conjugate (C) were obtained after dispersion into potassium bromide.

the bands' intensity, in the range of 1090 to 950 cm^{-1}; it was (Figure 3C) due to reactions between QKLI and

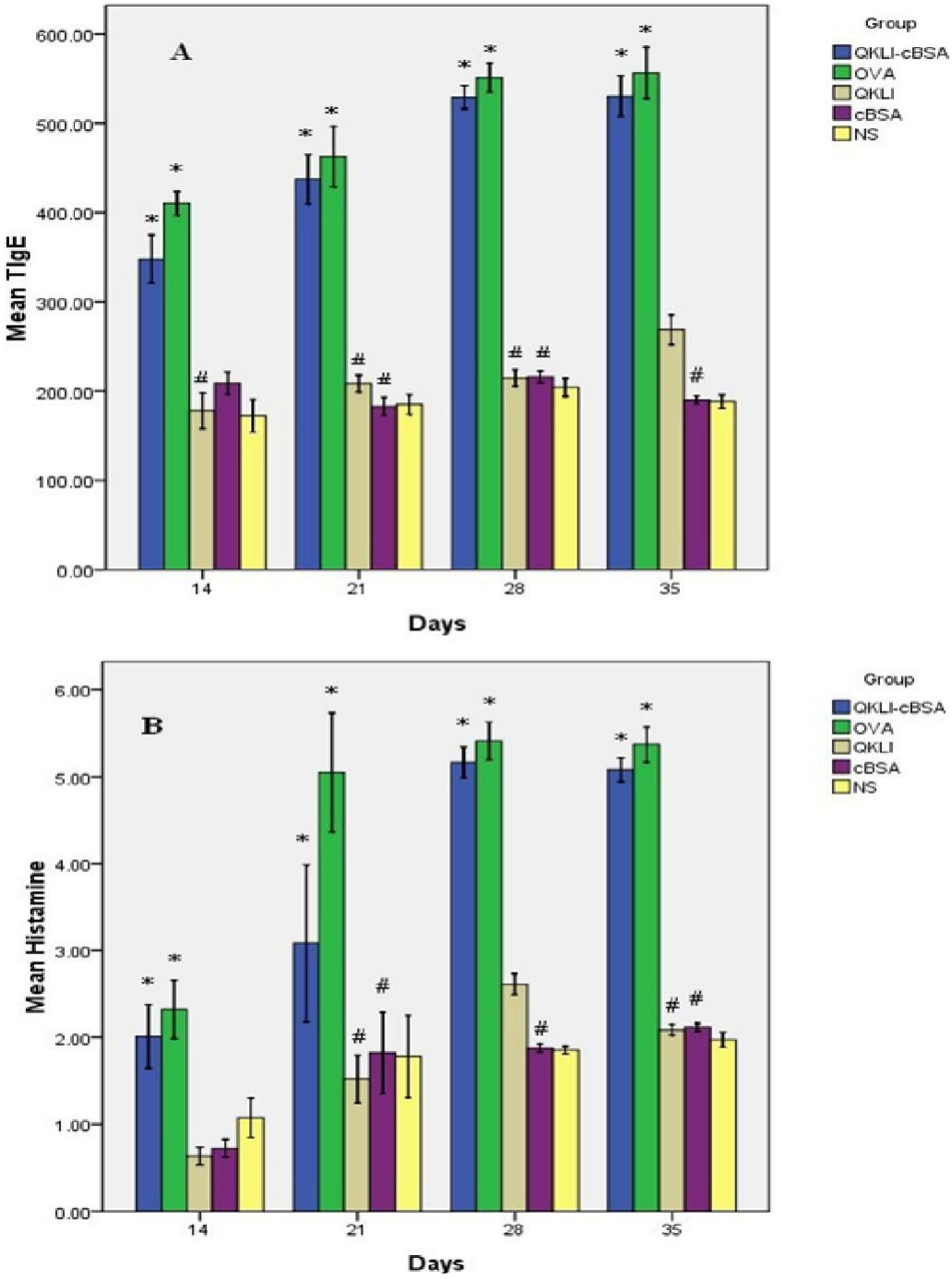

Figure 4. Effect of treatments on total IgE and histamine levels in mice serum. (A) Total IgE in serum. (B) Histamine in serum *(P<0.05), significantly different for groups compared with negative control. # (P>0.05), have no significant difference for groups compared with negative control.

primary amine, which depressed vibration of R-CH_2-NH_2.

Total IgE and histamine in mice serum

The levels of total IgE in mice serum were measured by ELISA. QKLI-cBSA and OVA treatments significantly increased total IgE levels in serum compared to the negative control (NS group) at different times (P<0.05) (Figure 4A). QKLI and cBSA treatments have no significant differences compared with negative control (NS group) (P>0.05) (Figure 4A).

The levels of histamine in mice serum were measured by ELISA. QKLI-cBSA and OVA treatment significantly

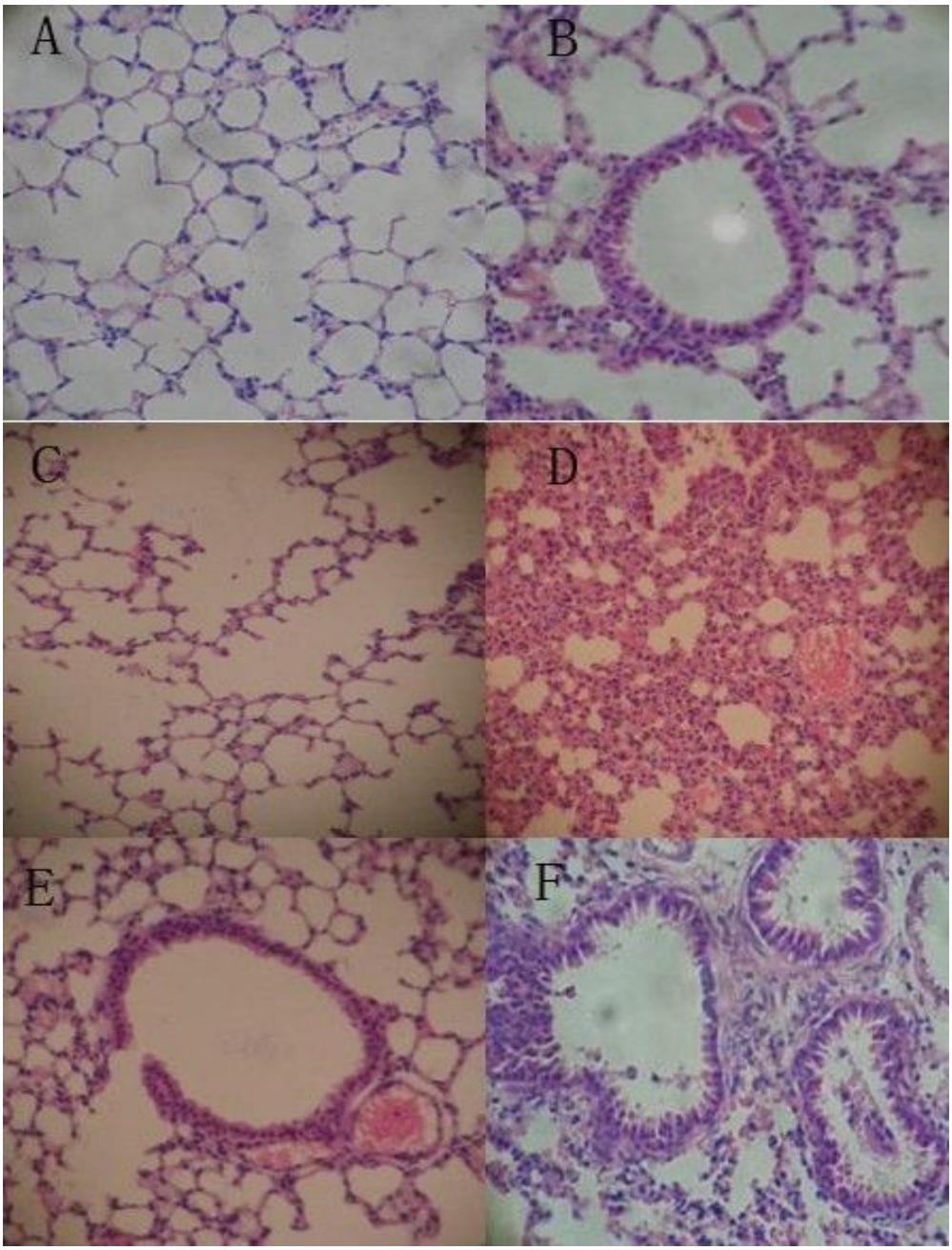

Figure 5. Histological evaluation of mice lungs treated with NS and QKLI-cBSA with hematoxylin and eosin staining (H&E × 200). (A) and (B) Negative control lung tissue. (C) QKLI-cBSA treated mice lung showed fusion of pulmonary alveoli. (D) QKLI -cBSA treated mice lung showed hyperemia. (E) QKLI-cBSA treated mice lung showed injury of trachea. (F) QKLI-cBSA treated mice lung showed neutrophilic cell infiltration.

increased histamine levels in serum compared to negative control (NS group) at different times (P<0.05) (Figure 4B). QKLI and cBSA treatments have no significant differences compared with negative control (NS group) (P>0.05) (Figure 4B).

These results suggest that total IgE and histamine are two of the major mediators involved in the anaphylaxis in this model.

Histology

QKLI-cBSA conjugate induced significantly acute injury in mice lung, as seen with hematoxylin and eosin staining. Blank lung tissue was seen in Figure 5A and B**.** QKLI-cBSA showed a considerable change in tissue structure due to acute injury, which can be demonstrated by fusion of pulmonary alveoli (Figure 5C), hyperemia of lung

(Figure 5D), injury of trachea (Figure 5E) and neutrophilic cell infiltration (Figure 5F).

Conclusion

Hapten is a class of LMWC and its immunogenicity is obtained only by coupling with some carrier protein (Roitt, 2001) . However, the haptens in QKLI were unknown and might have no available common functional groups (amines, carboxylates, sulfhydryls, etc.) except active hydrogens. Therefore, choosing a suitable carrier protein seems to be extremely important. Taking the aforementioned into consideration, we prepared an ideal carrier protein, cBSA. An active hydrogen-containing compound can be condensed with formaldehyde and an amine of cBSA in the Manncih reaction (Aime et al., 2004; Nobles and Potti, 1968), resulting in a stable conjugate. In this work, we found cBSA was an ideal carrier for unknown haptens in QKLI, indicating that it might be suitable for detecting unknown allergens in compound preparations and TCMIs.

The former preparation (Chu et al., 1982; Youxiang et al., 2007; Yi et al., 2009; Feng et al., 2010) for conjugate did not have any strategies of separation or purification. Furthermore, we firstly separated and purified the QKLI-cBSA conjugate by cationic ion exchange method, which was beneficial to eliminate the interference of impurities. The routine method of identifying the cationization of carrier protein was isoelectric focusing-polyacrylamide gel electrophoresis (IEF-PAGE) (Jean et al., 1999). In our work, the cationized carrier protein and the QKLI-cBSA conjugate were monitored by FT-IR spectroscopy, which was an effective tool to inspect the structural modifications in molecules by interpreting the position of wavenumbers and the variable transmittance in characteristic waveband. FT-IR spectrometry, an effective and convenient method, might be an alternative method to verify the cationization of protein.

The Balb/c mice were selected as such mice are high IgE responders, equivalent to an atopic phenotype (Baeza and Zubeldia, 2007; Nierkens and Pieters, 2005). On the one hand, Ian et al. (2004) measured the total and Ag-specific IgE in OVA-sensitized Balb/c mice serum by using ELISAs, discovered a strong and consistent relationship between them. On the other hand, IgE specific ELISAs require extensive optimization for each antigen examined and generally yield data expressed in arbitrary units unless monoclonal antibody (mAb) of similar affinity (that is, those that exhibit slopes parallel to that of polyclonal responses throughout the titration curve) are generated and made available in sufficient quantity to act as a standard. As soon as allergen binds to the receptor-bound IgE antibodies, stimulation of mast cells and release inflammatory mediators (Histamine and tryptase, etc) occur with minutes and results in the immediate development of clinical signs of anaphylaxis (Janos, 2005; Kinet, 1999).

Therefore, we measured the total IgE and histamine in Balb/c mice serum to judge whether the mice were sensitized by QKLI-cBSA or not.

In this study, we successfully cationized the nBSA and prepared QKLI-cBSA conjugate. We immunized the BALB/c mice with subcutaneous injection of QKLI-cBSA plus alum. After immunization, we verified the model by analyzing the total IgE and histamine levels in serum. In addition, we evaluated the lung reactions by analyzing lung pathologic changes. Finally, we successfully established a murine model of IgE-mediated Qingkailing injection anaphylaxis. The model might be enlightening allergen researchers on detecting allergens in QKLI and other Traditional Chinese Medical Injections (TCMIs). On the one hand, the protocol is expected to be applied in preparing QKLI-specific antibodies and detecting allergens in QKLI. On the other hand, the protocol can also be applied in establishing anapylaxis animal models of other TCMIs.

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