

## PRESENTATION OF RECEPTOR-CONTACTING LOOP OF HUMAN IGE ON THE HBCAG PARTICLES

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**Abstract:** Immunoglobulin of class E (IgE) plays a central role in allergies and allergic asthma. Therapeutic anti-IgE vaccine is expected to elicit autologous antibodies targeting the endogenous IgE molecules and neutralize allergic mechanism. Virus-like particles (VLPs) are a form of immunogens capable of breaking self-tolerance and represent an attractive platform for designing the therapeutic vaccines against self-antigens, such as the IgE. We designed VLPs from the core protein of hepatitis B virus (HBcAg). Arrays of the receptor-contacting epitopes of the human IgE were engineered on the VLP surfaces.

**Objectives:** Primary objective of this study was to develop IgE-epitope antigen that induces antibodies against the receptor-contacting epitope on the human IgE molecule.

**Methods:** Genes encoding the empty carrier and HBcAg with insertion of IgE epitope were constructed de novo and cloned into pET expression vectors. Ultracentrifugation in sucrose gradients and size exclusion chromatography (SEC) were used to purify VLPs. Electron microscopy and dynamic light scattering were used for proof of the VLPs appearance. Mice were immunized, and western blots were conducted with immune sera to reveal the presence of the induced anti-(human)IgE antibodies. Titers of the anti-IgE were measured in ELISA.

**Results:** Two types of plasmid constructs for expression of the VLPs carrying IgE epitope arrays were produced. One type represents an insertion of an immunogenic peptide into HBcAg by construction of a contiguous fusion protein in which the peptide is flanked with sequences of the carrier. The other type is different in that the carrier protein is "split" into two polypeptide chains which upon expression remain associated in a stable VLP-forming subunit. The splitting was achieved by construction of a bicistronic RNA (s.c. "SplitCore" technology). The obtained IgE-epitope antigens with contiguous sequences appeared to be incapable of formation of VLPs. On the contrary, the split core protein efficiently formed VLPs. Immunization of mice with the VLPs presenting the IgE Cε3 FG loop resulted in the development of high titers of antibodies recognizing human IgE in ELISA.

**Conclusion:** Recombinantly expressed VLPs presenting the IgE Cε3 FG loop elicit the anti-IgE antibodies upon immunization. Application of the SplitCore technology to construct IgE-epitope antigens can result in a pan-allergy anti-IgE vaccine.

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### Introduction

B-cell epitope-based vaccine is a concept involving artificial antigen, which comprises one or more precisely defined antigenic determinants or epitopes taken out from the natural antigen and embedded into a suitable carrier (Castelli et al., 2013). This concept suggests that properly selected epitope embedded in a heterologous carrier retains its native structure and the ability to elicit immune response equivalent to the response to natural antigens or even stronger. The epitope-based vaccines have an important advantage, which is the ability to elicit immune response against a precisely defined antigenic determinant.

Virus-like particles (VLPs) represent an efficient self-adjuvanting vaccine platform (Rivera-Hernandez et al., 2013). Many structural proteins of viruses, which are naturally involved in formation of virions, retain their ability to assemble into virus-like structures upon recombinant expression, i.e. in the absence of viral genomes or viral replicative machinery (Srinivasan, Rastogi, Ayyavoo, & Srivastava, 2014). VLPs are potent immunogens capable of priming T cell-dependent responses, and also induce T cell-independent antibody responses (Brun et al., 2011). Of particular importance is the fact that presentation of epitopes in a form of highly repetitive and ordered (quasi-crystalline) array on the surface of VLPs may overcome B cell unresponsiveness toward self-antigens (Chackerian, 2010).

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Among proteins exerting a VLP-forming capacity, core protein of hepatitis B virus (HBV) is particularly well-characterized. The HBcAg is a highly attractive VLP carrier because of its small size and high immunogenicity (Whitacre, Lee, & Milich, 2009). The HBcAg forms particles resembling nucleocapsids of HBV, although devoid from viral genome. The particle has spherical symmetry and present in two forms, one form composed from 120 HBcAg dimers (triangulation number T4; diameter ~34 nm), and the other composed from 90 dimers (T3; diameter ~30 nm). The HBcAg VLPs are naturally highly immunogenic, and recombinant fusion proteins with the HBcAg backbone often retain high immunogenicity of the carrier (Newman, Suk, Cajimat, Chua, & Shih, 2003). Predominantly, alpha-helical 3D structure of the HBcAg contains an alpha-hairpin, which participates in the HBcAg dimer formation. Two hairpins compose a four-helix bundle, which presents itself as a spike protruding from particle's surface. The tip of the spike, called the major immunodominant region (MIR), corresponds to the HBcAg sequence between residues 78-82 (Yu, Jin, Jih, Shih, & Zhou, 2013). The MIR is a preferred place for insertion of the B-cell epitopes because inserted sequences are exposed on the outer surface of VLPs (Roose, De Baets, Schepens, & Saelens, 2013). The HBcAg VLP platform ensures multiplicity of presentation of the epitope (s.c. "epitope array") and optimal spacing between the individual epitopes in an array.

Allergic conditions are among the most common chronic disorders in developed countries (Sabban et al., 2013). The pathological mechanism of the conditions mentioned, which are generally referred to as an immediate (type I) hypersensitivity, involves hyperproduction of immunoglobulin of class E (IgE) and a series of pathological reactions involving the IgE. The IgE antibodies attach to effector cells (i.e. mast cells and basophils) through interaction with high affinity receptor FcεRI. Subsequent exposure to allergen leads to binding of the multivalent allergen to the specific IgE on cell membranes and cross-linking of the receptor molecules. Such cross-linking in turn triggers activation of the effector cells, release of mediators of vascular permeability, and smooth muscle contraction. Activation of the effector cells subsequently causes late reactions, including release of inflammatory mediators, which support chronic inflammation. Crucial role of the IgE in the pathological mechanism of allergies and asthma makes IgE an attractive target for medical intervention. Passive anti-IgE immunization via injection of a therapeutic anti-IgE monoclonal antibody (mAb) Omalizumab (Xolair) results in a clinically proven beneficial effect in asthmatic patients. A different promising treatment approach is an active immunization of patient with a therapeutic pan-allergy vaccine, which is yet to be developed. Extensive efforts were made to develop antigens capable of eliciting autologous IgE-blocking antibodies of classes G and A (anti-IgE IgG and IgA) (Lee, 2014).

In this article, we describe a technology of presentation of a particular IgE epitope on surface of the HBcAg VLPs. These VLP antigens present arrays of the receptor-contacting IgE epitope (FG loop of a third domain (Cε3) of the human IgE heavy chain).

## **Materials and methods**

### ***Bacterial strains, plasmids, and synthetic genes***

The *E. coli* strain JM109 was used for genetic engineering; BL21(DE3) was used for expression of the recombinant proteins. Gene encoding the HBcAg (subtype ayw) was produced in our laboratory utilizing method of the de novo gene synthesis in PCR. The synthesized HBcAg gene was used to develop genes of antigens carrying insertions of a peptide from a receptor-contacting site of IgE.

### ***Protein expression and purification of the VLPs***

BL21(DE3) cells transformed with expression constructs were inoculated into 1 L of LB medium with ampicillin (100 mg/ml). Flasks were kept on a shaker at 37°C until cultures reach optical density at 600 nm 0.6-0.8. Upon this time, flasks were transferred to a shaker set at room temperature (RT), and

IPTG was added to a final concentration of 1 mM. Induced cultures were incubated at RT overnight. Bacterial cells were collected by centrifugation. The pellets were resuspended in a lysis solution (20% sucrose, 20 mM HEPES pH 7.5, 5 mM EDTA, 0.1% Triton-X100, 1 mg/ml lysozyme, 10 mg/ml DNase I, 100 mg/ml RNase, 0.2 mM PMSF). The suspensions were incubated at RT for 1 hour. Completeness of lysis was achieved by sonication (ten pulses, each pulse 20 sec long). Urea was added to a final concentration of 0.5 M, and solution was incubated on ice for 30 min. Debris was removed from lysates by centrifugation. The VLPs were sedimented through sucrose cushion (20% (w/w) sucrose solution prepared in HN buffer (50 mM HEPES pH 7.5; 150 mM NaCl)) using rotor SW28 (28000 rpm, 12-16 hours). The pellets were resuspended in 2 ml of the HN buffer and filtered through 0.22 µm syringe filters. The samples were loaded on top of preformed sucrose step gradients (10%-60%) in rotor SW41. Ultracentrifugation in gradient proceeded at 35000 rpm, 12-16 hours. The established gradients were fractionated into 1 ml fractions.

#### ***Size exclusion chromatography (SEC)***

Samples from sucrose gradients were loaded on Superose 6 10/300 GL column connected to an FPLC system AKTA Purifier 10 (GE Healthcare). The column was pumped with the HN buffer at a flow rate 0.3 ml/min. Elution profile was recorded at a wavelength of 280 nm. Chromatographic peaks were collected using a fraction collector Frac-950. Protein concentration was determined using Bradford method.

#### ***Electron microscopy***

Droplets of samples were placed on a sheet of parafilm. Carbon-formvar-coated copper grids were allowed to float on the droplets coated side down. The grids were then transferred on a drop of 2% phosphotungstic acid pH 7.0. The grids were examined with a LIBRA 120 transmission electron microscope (Carl Zeiss) at magnifications 30000-100000X.

#### ***Immunization of mice***

Groups of five female BALB/c mice were used for immunizations. Baseline sera were collected and pooled. Control group was immunized with the empty carrier. Experimental group was immunized with the split core antigen (HBcAg-IgE(SC)). The immunization scheme included four subcutaneous injections: 25 mg of the VLP antigen in PBS mixed with a complete Freund's adjuvant for the first injection, and 25 mg of the antigen in PBS without adjuvants for the remaining injections. The mice were immunized subcutaneously on days 1, 7, 14, and 28. Blood was collected on day 42. Sera from individual mice were pooled within the control and experimental groups. Experiments were approved by the Animal Ethics Committee (AEC) in the National Center for Biotechnology (Astana).

#### ***Enzyme-linked immunosorbent assay (ELISA)***

In ELISA, to detect anti-IgE antibodies in mice sera, purified human immunoglobulin E was used as a capture antigen. The IgE (Abcam, Cat# ab65866) was diluted to the concentration of 5 mg/ml in a 50 mM sodium carbonate buffer pH 9.6. Aliquots of the IgE solution (100 µl) were dispensed into wells of a 96-well plate (Costar, USA), and the plate was incubated overnight at 4°C. PBST was used as a washing buffer, and a PBST+1% BSA was used as a blocking buffer and a diluent for serum. After blocking with the PBST+BSA for 1 hour at 37°C, serial dilutions of sera were dispensed into wells. Plate was incubated for an additional 1 hour at 37°C. Upon three washes with PBST, a horseradish peroxidase conjugated anti-mouse IgG antibody (Sigma, Cat# A9044) was applied, diluted 1:10000 in PBST+BSA. Following 1 hour of incubation at 37°C, the plates were washed 3 times with PBST and then 3 times with deionized water. The liquid TMB substrate system for ELISA (Sigma, Cat# T0440) was added. Color reaction was stopped by addition of 100 µl of 2M H<sub>2</sub>SO<sub>4</sub>. Absorbance values were read at 450 nm.

**Results**

To develop expression constructs, the synthetic genes were cloned into a polylinker of plasmid pET22/28, which was created in our laboratory from plasmids pET22b and pET28c (Novagen). The pET22/28 has sequence of the plasmid pET22b except for a region of polylinker, which fragment XbaI-XhoI is derived from the plasmid pET28c. Sequences of the recombinant expression products are presented in Figure 1. In native HBcAg, the C-terminal protamin-like domain is responsible for binding of nucleic acids but is not required for the VLP assembly. Thus, to utilize only the VLP-forming capacity of the HBcAg but not the nucleic acid-binding property, we truncated the sequence of the recombinant antigen at Val149. We produced IgE epitope-containing antigens by engineering of the human IgE-derived sequence GETYQSRVTHPHLPRALMRSTTK into the modified HBcAg. Sequence of the IgE epitope is flanked with flexible glycine-rich linkers, G4E2SG4T and TG4SE2G4 (Figure 1). The linkers are intended to minimize interference between tertiary structure of the backbone protein and that of the heterologous insert.

Figure 1: Sequences of recombinant proteins. His-tags are added to all N-termini (depicted with dashed line above alignment). The HBcAg is truncated at Val<sub>149</sub>. In IgE-epitope antigens HBcAg-IgE, HBcAg-IgE(Cys), and split core HBcAg-IgE(SC), the Pro-Ala sequence is replaced with heterologous insert. The replaced residues Pro<sub>79</sub> and Ala<sub>80</sub> are marked with asterisks. Within the inserts, sequence corresponding to the IgE peptide is underlined. The split core antigen HBcAg-IgE(SC) consists of two chains shown in separate lines of alignment, namely the HBcAg-IgE(SC)N and HBcAg-IgE(SC)C.

HBcAg	.....	MGHHHHHSGGMDIDPYKEFGATVELLSFLPSDFPFSVRDLLDTASALYREALESPHECSPHHTALRQAILCWGELMTLA	80
HBcAg-IgE		.....	80
HBcAg-IgE (Cys)		.....	80
HBcAg-IgE (SC) N		.....	80
HBcAg-IgE (SC) C		-----	-
HBcAg	**	TWVGVNLEDPA-----SRDLVVSIVNTNMGLKFRQLLW	113
HBcAg-IgE		.....GGGGEESGGGGT-GETYQSRVTHPHLPRALMRSTTK-TGGGGSEEGGG.....	158
HBcAg-IgE (Cys)		.....GGGGEESGGGGTCGETYQSRVTHPHLPRALMRSTTKCTGGGGSEEGGG.....	160
HBcAg-IgE (SC) N		.....GGGGEESGGGGTCGETYQSRVTHPHLPRALMRSTTKC-----	126
HBcAg-IgE (SC) C		-----M.....	23
HBcAg		FHISCLTFGRETVIEWLVSFGVWIRTPPAYRPPNAPILSTLPETTVV	160
HBcAg-IgE		.....	205
HBcAg-IgE (Cys)		.....	207
HBcAg-IgE (SC) N		-----	-
HBcAg-IgE (SC) C		.....	70

Source: Authors

Three different variants of insertion were tried. The first variant, namely the HBcAg-IgE is the HBcAg with insertion of the IgE peptide into the MIR site (Figure 1). In the second variant, namely the HBcAg-IgE(Cys), the Cys residues were engineered, which immediately flank the IgE peptide (Figure 1). Formation of disulfide bond between the flanking Cys residues is thought to provide an additional conformational constraint (cyclization) to the IgE epitope. The third variant, namely the HBcAg-IgE(SC) differs from the previously described variants in that the HBcAg-IgE, and HBcAg-IgE(Cys) are contiguous polypeptide chains. The HBcAg-IgE(SC) is an assembly of two different polypeptide chains. The HBcAg-IgE(SC) construct has been developed utilizing a “split core” technology described by Walker, Skamel, & Nassal (2011).

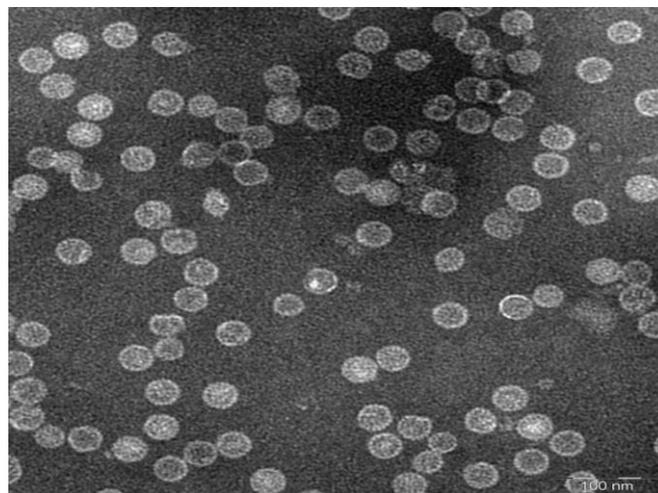
This approach makes it possible to produce the HBcAg derivative in a form of two polypeptide chains translated from a bicistronic mRNA. The bicistronic mRNA has two open reading frames separated by engineered internal ribosome binding site (iRBS). This iRBS is a Shine-Dalgarno sequence, whose presence leads to a non-dissociation of ribosome upon completion of translation of the first ORF and initiation of translation of the second ORF. Two ORFs encode two protein chains, designated according to their correspondence to the N- or C-terminal parts of the SplitCore as HBcAg-IgE(SC)N

and HBcAg-IgE(SC)C (Figure 1). Upon translation, the HBcAg-IgE(SC)N and HBcAg-IgE(SC)C remain associated in a stable complex, which is structurally similar to one HBcAg molecule. More importantly, this complex is capable of formation of VLPs.

#### *Visualization of VLPs using electron microscopy*

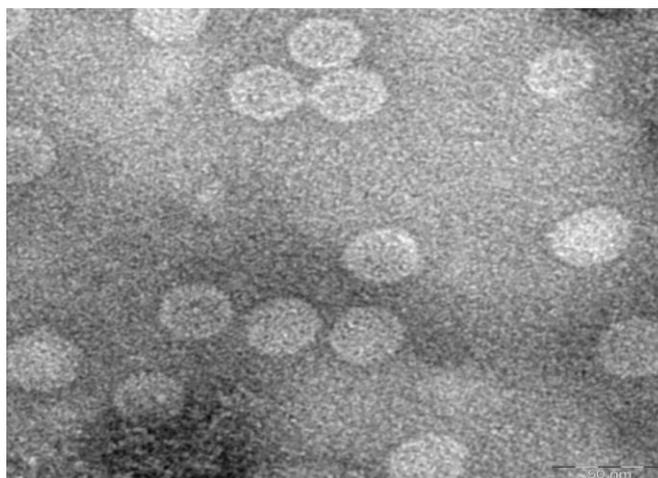
When sample of the HBcAg was examined by electron microscopic examination after negative staining, the abundance of VLPs with diameters of approximately 32 nm was evident (Figure 2). Also, spherical VLPs with similar size were observed in sample of the split core IgE-epitope antigen HBcAg-IgE(SC) (Figure 3). Observed diameters of the VLPs (32-35 nm) are consistent with data from the DLS analysis. No particles with regular morphology were found in samples of the IgE antigens with contiguous (not split core) sequences HBcAg-IgE and HBcAg-IgE(Cys).

Figure 2: Electron microscopy of VLPs in sample of the empty carrier HBcAg. Negative stain, scale bar: 100 nm.



Source: Authors

Figure 3: Electron microscopy of VLPs in sample of the SplitCore IgE-epitope antigen HBcAg-IgE(SC). Negative stain, scale bar: 50 nm.

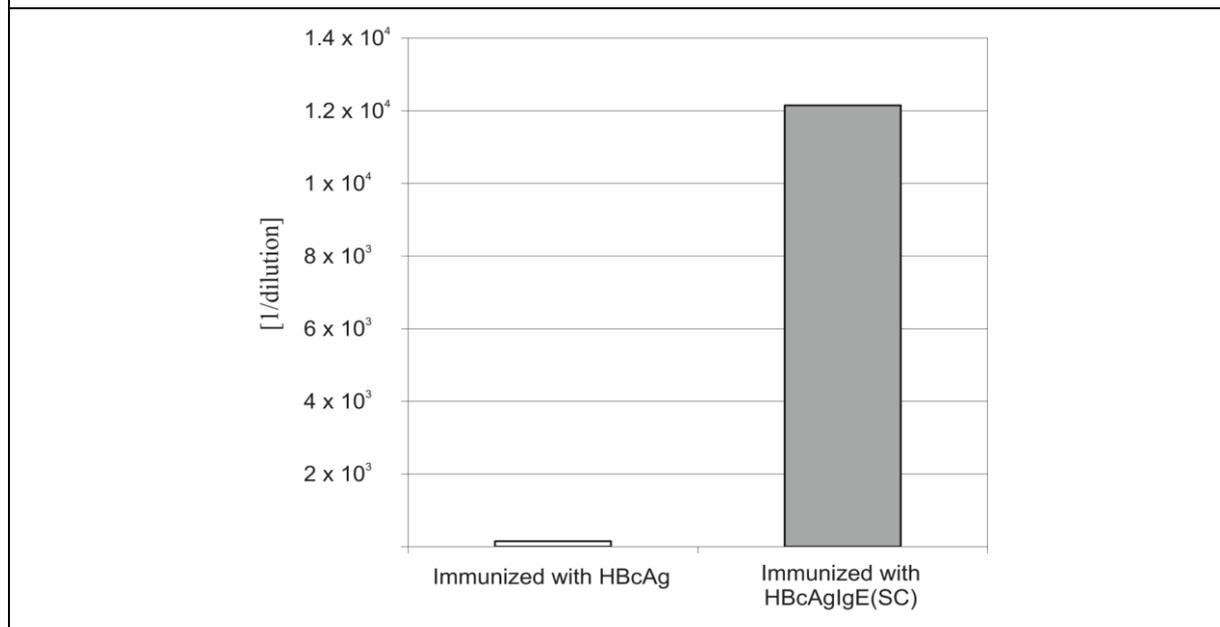


Source: Authors

***ELISA detects induced anti-(human)IgE antibodies in mice immunized with the SplitCore antigen***

Direct ELISA was used to determine antigenicity of the IgE epitope presented on the surface of the VLPs. Human immunoglobulin IgE was clearly recognized by antibodies elicited by immunization with the IgE-epitope VLP antigen HBcAg-IgE(SC). Titer of the anti-(human)IgE antibodies in the pooled mouse serum reached 1:12150 after four immunizations (Figure 4).

Figure 4: Recognition of the human immunoglobulin E by induced anti-(human) IgE antibodies in ELISA. Three samples of the pooled serum were tested for reactivity with the human IgE: 1) serum from mice immunized with the split core IgE-epitope antigen HBcAg-IgE(SC); 2) serum from mice immunized with the empty carrier HBcAg; 3) baseline (pre-immunization) serum. The titers represent dilutions of immune sera, which produced absorbances greater than that of the baseline sera.



Source: Authors

**Discussion**

Like viruses, VLPs are highly immunogenic. Also, like viruses, VLPs are potent inducers of B- and T-cell responses. HBcAg is one of the most attractive VLP scaffolds because of small size, high immunogenicity, and large carrier capacity. It was found that the position of foreign epitope insertion into the HBcAg greatly influences the epitope immunogenicity. Insertions into the tip of protruding spike (MIR domain) are the most immunogenic.

In this study, we aimed to explore the VLP-forming property of the HBcAg to produce VLP antigens capable of eliciting antibodies against the receptor-contacting epitopes of human IgE. Antigens capable of overcoming the B cell tolerance and inducing anti-IgE blocking antibodies of IgG or IgA classes may be used in a pan-allergy immunotherapeutic vaccine. A task of this study prompts the selection of an IgE sequence for use as an epitope to which the humoral immune response targets. The search for the receptor-contacting linear or discontinuous IgE epitopes may be based on a crystal structure of the IgE/receptor complex. The FG loop of a third domain (Cε3) of the human IgE heavy chain plays a crucial role in the binding of the IgE-Fc to the FcεRI. Involvement of the FG loop in the receptor recognition is underscored by the fact that the binding site for the blocking antibody Omalizumab maps to the FG loop (Zheng et al., 2008).

We designed recombinant antigens utilizing the HBcAg as a carrier and the FG loop as an insert. One variant (HBcAg-IgE) has contiguous amino acid sequence with the insert flanked with long glycine-rich linkers. It is generally assumed that the flexible linkers facilitate correct folding of the fusion proteins. Sufficiently long flexible linkers were needed to preserve the ability of the carrier to form VLPs (Klamp et al., 2011). The second variant (HBcAg-IgE/Cys) has a contiguous sequence in which the insert is additionally flanked with adjacent Cys residues. The flanking Cys residues were introduced with an intention to cyclize the insert as was done to the FG loop sequence according to Wang et al. (2003). However, both variants of the IgE-epitope antigens with contiguous sequences did not produce VLPs, probably because of the structural incompatibility between the carrier and the inserts. The third variant, the split core antigen HBcAg-IgE(SC) is an example of a utilization of a novel VLP carrier. Unlike traditional HBcAg, which is a single polypeptide chain, the split core HBcAg is a complex composed of two different polypeptide chains. The two chains are produced during heterologous expression from a single bicistronic mRNA. Utility of the split core technology and design of the bicistronic mRNA were originally published by Walker et al. (2011). When applied as a carrier for presentation of the FG loop epitope, the split core HBcAg enabled the production of a significant portion of the antigen in a soluble (cytoplasmic) fraction. The VLPs composed from the HBcAg-IgE(SC) protein were isolated from the soluble fraction. Our data are in accordance with the results published by Walker et al. (2011) in that the N- and C-terminal parts of the split core HBcAg remain associated in one particle-forming subunit, and the free carboxyl terminus of the N-terminal part allows incorporation of complex epitopes without loss of the particle-forming capacity. Thus, the split core technology greatly widens the utility of the HBcAg as a carrier.

## Conclusion

We describe here a quick method for detecting the presence of the VLPs in samples using agarose gel electrophoresis in the gels containing dye brilliant cresyl blue. Our results show that fractions located in the center of 20-60% sucrose gradient, which produce sharp bands in NAGE, and which give characteristic VLP peak in SEC, actually contain VLPs as detected by electron microscopy and DLS method. Thus, three applied purification methods, namely the separation by density by the ultracentrifugation in gradients, the NAGE, and the SEC together constitute an effective technology for analysis of production and purification of the VLPs.

Our results indicate that immunization with the recombinantly expressed VLPs presenting the Cε3 FG loop epitope resulted in the generation of antibodies capable of recognition of human IgE. Further development of this strategy can result in a long anticipated pan-allergy anti-IgE vaccine.

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