

• VIRAL LIVER DISEASES •

# Construction and characterization of an experimental ISCOMS-based hepatitis B polypeptide vaccine

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## Abstract

**AIM:** To characterize the biochemical and immunological properties of an experimental ISCOMS vaccine prepared from a novel therapeutic polypeptide based on T cell epitopes of HBsAg, and a hepatitis B-ISCOMS was prepared and investigated.

**METHODS:** An immunostimulating complexes (ISCOMS)-based vaccine containing a novel therapeutic hepatitis B polypeptide was prepared by dialysis method, and its formation was visualized by electron microscopy and biochemically verified by SDS-polyacrylamide gel electrophoresis. Amount of the peptide within ISCOMS was determined by Bradford assay, and specific CTL response was detected by ELISPOT assay.

**RESULTS:** Typical cage-like structures of submicroparticle with a diameter of about 40nm were observed by electron microscopy. Results from Bradford assay showed that the level of peptide incorporation was about  $0.33\text{g}\cdot\text{L}^{-1}$ . At the paralleled position close to the sixth band of the molecular weight marker (3480kDa) a clear band was shown in SDS-PAGE analysis, indicating successful incorporation of polypeptide into ISCOMS. It is suggested that ISCOMS delivery system could efficiently improve the immunogenicity of polypeptide and elicit specific immune responses *in vivo* by the results of ELISPOT assay, which showed that IFN- $\gamma$  producing cells (specific CTL responses) were increased (spots of ISCOMS-treated group:  $47\pm 5$ ,  $n=3$ ; control group:  $5\pm 2$ ,  $n=3$ ).

**CONCLUSION:** ISCOMS-based hepatitis B polypeptide vaccine is successfully constructed and it induces a higher CTL response compared with short polypeptides vaccine *in vivo*.

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## INTRODUCTION

Infection of hepatitis B virus (HBV) is very common in China<sup>[1-25]</sup>, and nearly 100 million people have a persistent infection with HBV who are at risk of developing chronic hepatitis leading to liver cirrhosis and

hepatocellular carcinoma. Up to now, vaccination is a main way in prevention<sup>[26-36]</sup>. Based on our knowledge and work on epitopes of HBV natural nucleocapsids, using SGI O2 workstation and Insight II software modeling the configuration of natural HBV PreS2, HBsAg and HbcAg, we have screened out several novel HBV therapeutic polypeptides containing immunodominant B-, T helper (Th) and cytotoxic T lymphocyte responses (CTL) epitopes of HBV PreS2, HBsAg and HbcAg. It is well known that natural antigens and their dominant epitope peptides can not induce sufficient antigen-specific CTL responses *in vivo*, although they could pulse antigen-specific CTLs *in vitro*. Therefore efforts should be made to potentially promote or enhance their antigenicity so as to induce efficient immune responses including CTLs *in vivo*, among which utilization of appropriate adjuvants or delivery systems is a promising and useful strategy.

Elaborate work has demonstrated that ISCOMS, or immunostimulating complexes, first described by Sweden scientist Bror Morein and his colleagues in 1984, is a good vehicle for antigen presentation. Previously antigens used in earlier works in this complexes were isolated from crude molecular components of microbes while is prepared from phosphatidylcholine (or phosphatidylethanolamine), cholesterol and glucoside Quil A (also called Spikoside) and antigen molecule now, which are approximately 40nm submicroscopic cage-like particles (the size is similar to virus particle). ISCOMS formed in the absence of antigen molecules is termed ISCOMS matrix or empty ISCOMS compared to ISCOMS formed in the presence of antigen(s). Elaborate work has demonstrated that ISCOMS is a good vehicle for antigen presentation. Incorporation into ISCOMS not only allows protein antigens to induce strong antibody, major histocompatibility complex (MHC) class II-restricted T cell responses and mucosal immunity, but also allow antigens to enter the endogenous pathway of antigen processing to induce MHC class I-restricted CTL *in vivo*<sup>[37-39]</sup>. Immunization with antigens in ISCOMS induces protective immunity against a number of experimental infections, including influenza, toxoplasmosis, measles, feline leukemia, EBV infection, and herpes simplex<sup>[40-42]</sup>. An ISCOMS-based vaccine against equine influenza was produced and sold by Iscotec AB in Sweden in 1988. Compared with liposomes, ISCOMS structure is much more rigid, three-dimensional with marked symmetry, which is extremely stable under many conditions, including in the intestine, and may be present for long periods of time intra- and extracellularly in lymphoid tissues. On the basis of our previous work on the design and synthesis of HBV epitope-based vaccines, choosing a polypeptide containing both Th and CTL epitopes of HBV as a model antigen, an experimental ISCOMS-based vaccine was constructed and prepared, and its biochemical and immunological properties were then investigated and discussed in this study.

## MATERIALS AND METHODS

### Reagents and preparation

Phosphatidylcholine, cholesterol, and decanoyl-N-methyl-glucamide (Mega-10) were all from Sigma. Quil A was kindly provided by Dr. Erik B Lindblad. SDS molecular mass marker (2500-17000u) was from Sigma (MWM-100). Hepatitis B polypeptide, glycopeptide and lipopeptide were synthesized by the solid-phase method with an automated peptide synthesizer (431A, Applied Biosystems, Foster City, CA).

### Lipid mixture stock solution

Phosphatidylcholine and cholesterol( $10\text{g}\cdot\text{L}^{-1}$  each) were fully dissolved in  $200\text{g}\cdot\text{L}^{-1}$ (in distilled water) Mega10, aliquot and store at low temperature until required.

### Bradford solution

Dissolve 100mg Coomassie blue G and 30mg SDS in 50mL  $950\text{mL}\cdot\text{L}^{-1}$  ethanol. Add 100mL of  $850\text{g}\cdot\text{L}^{-1}$  orthophosphoric acid and make up to 1L with water. Filter before use.

### Preparation of an experimental ISCOMS-based vaccine from a therapeutic hepatitis B short polypeptide

To a solution of hepatitis B polypeptide( $1\text{g}\cdot\text{L}^{-1}$ ) and Quil A( $1\text{g}\cdot\text{L}^{-1}$ ), add  $100\mu\text{L}$  of lipid mixture stock solution and mix thoroughly. After reaction at room temperature for 2h, the mixture was transferred to a dialysis bag, dialyzed 24h at room temperature, then at  $4^{\circ}\text{C}$  for another 48h. To confirm the presence of typical structure of ISCOMS, a small aliquot( $\sim 80\mu\text{L}$ ) was negatively stained and examined by electron microscopy analysis. Sterilize by filtration through a  $0.22\mu\text{m}$  filter.

### Amount of polypeptide incorporated into ISCOMS determined by Bradford method

To each  $50\mu\text{L}$  sample of ISCOMS and to each  $50\mu\text{L}$  dilution of BSA add 1mL Bradfords solution. Incubate 5min at room temperature. Measure the  $A_{595}$  of each sample and BSA standard. Plot a standard curve from the BSA values and determine the protein concentration of the ISCOMS by interpolation from this curve.

### Incorporation of short peptide into ISCOMS examined by peptide SDS-PAGE analysis

**Table 1** Formulation of gels

Components	Stacking gel (mL)	Spacer gel (mL)	Separating gel (mL)
Acrylamide solution	5.00	3.05	1.00
Gel buffer	5.00	5.00	3.10
Glycerol	1.60	—	—
Water	3.40	6.95	8.40
Total	15.00	15.00	12.50

Prepare gels as indicated in Table1. After the gel has set, allow to equilibrate by leaving overnight at  $4^{\circ}\text{C}$ . Remove the comb and rinse wells with water, then with Cathode Buffer. Load the samples and molecular weight marker. Electrophoresis condition: constant current at 20mA for 1h and 30mA for 4-6h (the marker dye is within 1cm of the anodic end of the gel). Immerse in the fixative solution for 30min. Transfer to staining solution for 1h and destaining solution for 2h, renewing the solution every 30 minutes. The gel now is ready for visualization, analysis or qhotography.

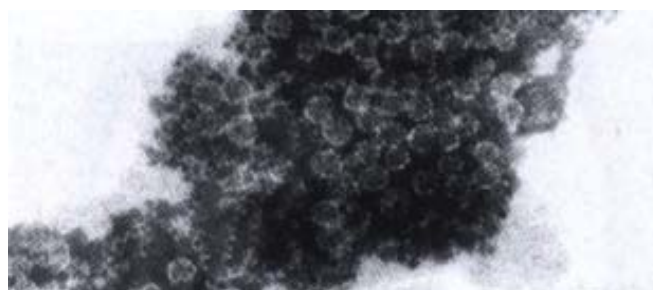
### ELISPOT assay

Female Balb/c mice, obtained from the Animal Research Center of Academy of Military Medical Sciences(Beijing, China) were sc injected to the hind footpad with hepatitis B polypeptide-ISCOMS and hepatitis polypeptide( $5\text{nmol}$  each) alone. After 7d of the first priming, the animals were boosted and lymph nodes were removed 7d later to prepare single cell suspension for the ELISPOT assay, which was performed according to the instruction of the murine IFN- $\gamma$  ELISPOT kit(Diaclone, France).

## RESULTS

### Typical structure of ISCOMS prepared from hepatitis B polypeptide by electron microscopy

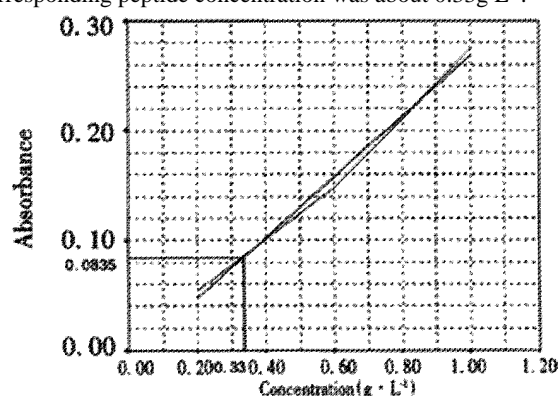
Hepatitis B-ISCOMS unique structure was examined by electron microscopy, which showed uniform honeycomb-like open structure composed of several subunits and confirmed the formation of ISCOMS (Figure 1).



**Figure 1** Visualization of hepatitis B-ISCOMS by electron microscopy.

### Amount of hepatitis B polypeptide incorporated into ISCOMS

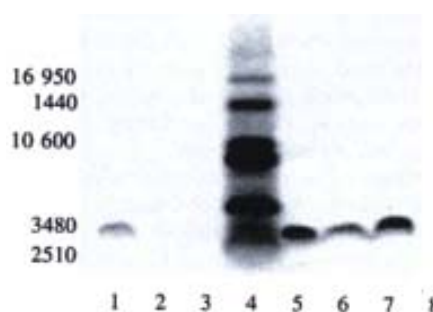
Standard curve was shown as Figure 2( $r=0.9968$ ). The mean  $A_{595}$  value of ISCOMS was 0.0835. According to the standard curve, the corresponding peptide concentration was about  $0.33\text{g}\cdot\text{L}^{-1}$ .



**Figure 2** Standard curve of Bradford assay

### SDS-PAGE analysis of ISCOMS

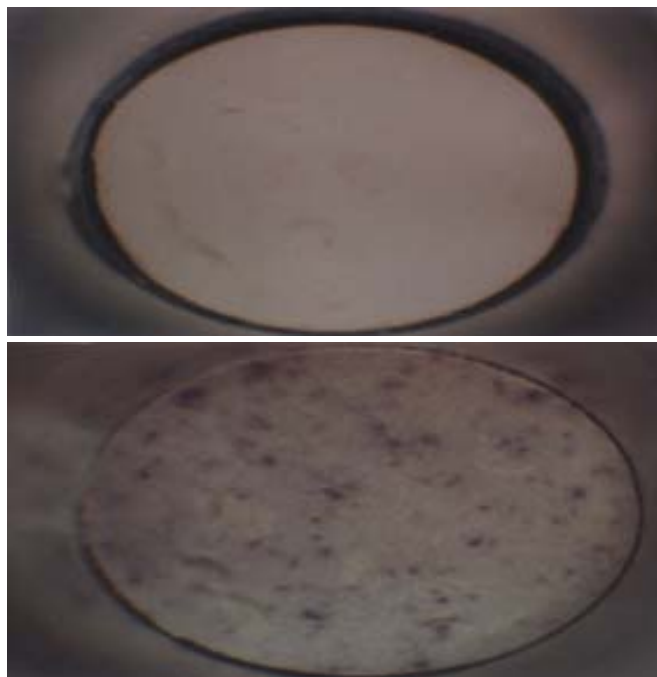
In the SDS-PAGE separation of ISCOMS prepared from different hepatitis B polypeptides we observed that at the paralleled position close to the sixth band of the molecular weight marker there was a clear band indicating the peptide incorporated into ISCOMS(Figure 3). Compared to the polypeptide sample, it was at the similar position but somewhat with a slight tailer, which might be affected by other components in the ISCOMS.



**Figure 3** SDS-polyacrylamide gel separation of hepatitis B polypeptide-ISCOMS. 1.Hepatitis B peptide(30)-ISCOMS; 2.ISCOMS matrix; 3.Hepatitis B lipopeptide-ISCOMS; 4.Molecular weight marker; 5.Hepatitis B peptide(30)(as control); 6,7.Hepatitis B peptide(30)-ISCOMS; 8.Hepatitis B glycopeptide -ISCOMS.

### IFN- $\gamma$ linked-CTL activity of Hepatitis B polypeptide-ISCOMS determined by ELISPOT assay

Swelling of lymph nodes were obvious in ISCOMS treated group. In ELISPOT assay, significantly improved IFN- $\gamma$  linked cytotoxic T lymphocyte(CTL) proliferation response to hepatitis B polypeptide was observed in HBP-ISCOMS group compared to the polypeptide control group(spots of B:  $47 \pm 5$ ,  $n=3$ ; A:  $5 \pm 2$ ,  $n=3$ , respectively)(Figure 4).



**Figure 4** ELISPOT results showing improved specific CTL responses induced by hepatitis B polypeptide incorporated into ISCOMS (B) compared with the polypeptide (A)

## DISCUSSION

Studies in immunological mechanisms and T cell function have demonstrated that class I major histocompatibility complex(MHC)-restricted cytotoxic T lymphocytes(CTL) play a critical role in the control of intracellular pathogens and tumor cells growth, but the problems of CTL responses induced *in vivo* and cell-mediated immunity have not been solved yet. Exogenous soluble antigens are not allowed to enter the endogenous pathway necessary for MHC class I-restricted presentation, therefore are not capable of stimulating MHC class I-restricted CD8<sup>+</sup> CTL responses. Obviously, intracellular expression and synthesis of antigens by infection or through pathological/physiological genes could lead to MHC class I restricted-representation pathway, but vaccination of infectants or transfected cells might lead to certain diseases, thus development of adequate novel vaccine adjuvants, especially those themselves are not immunogenic and could present non-replicable soluble antigens to the endogenous pathway and are capable of inducing MHC class I restricted-CTL responses, are badly in demand.

ISCOMS technique is just one of the novel adjuvanted-vaccine systems to meet this demand. Since the first description of ISCOMS appeared more than a decade ago, ISCOMS has been widely used to generate antigen-adjuvant complex in vaccine development, especially in viral antigen studies to promote immune responses. ISCOMS has a unique ability to provoke a full range of immune response to protein antigens, which is efficient after both parenteral and oral immunization. It has a unique ability to allow the antigen molecules to enter the endogenous pathway for antigen processing, which in turn to provoke MHC class I-restricted CTL. It is safe and stable, prepared

in mild conditions. Furthermore, as ISCOMS is a non-viable adjuvant vesicle and is not immunogenic and antigenic itself, it could enhance antigen specific immune responses, but not unwanted immunity. Different antigen molecules are able to produce vaccine-adjuvant complex with ISCOMS matrix after proper modification, so are useful and promising in various vaccine design.

Preparations of experimental ISCOMS-based vaccines, have been done with large number of whole microbe or isolated from microbe, especially viruses such as HIV<sup>[43-46]</sup>, influenza virus<sup>[38,47-49]</sup>, EBV<sup>[41]</sup>, HSV<sup>[42,50]</sup> and Measles<sup>[40,51]</sup>. The work with the major S gene products (HBsAg) of the hepatitis B virus genome has been reported, but not with epitope-based hepatitis B polypeptide. In our study, on the basis of the molecular design and synthesis of therapeutic hepatitis B peptides previously, different hepatitis B polypeptides were investigated for their abilities to incorporating into ISCOMS.

Formation of typical structure of ISCOMS was verified by electron microscopy. Adequate proportion of the components, extensive dialysis and purification by density gradient ultracentrifugation when necessary, are important factors involved in the preparation of ISCOMS. The presence of typical cage-like microparticles visualized by electron microscopy is a simple and direct method to examine the formation of ISCOMS, but ISCOMS matrix also shows similar structure as ISCOMS containing antigen molecules, so other methods are required to verify the presence of antigen component. Results from Bradford assay suggested that ISCOMS was successfully formed with polypeptide(30) and other components. In our study, polypeptide incorporated into ISCOMS was about 0.33g·L<sup>-1</sup>(incorporation rate 33%). In addition, we observed that the short polypeptide which was easily degraded in a couple of days even at 4°C, was stable while stored at 4°C for months without notable degradation, indicating markedly improved stability of antigen peptide after the formation of hepatitis B short polypeptide-ISCOMS.

SDS-PAGE separation of short polypeptide showed an apparent band close to 3480Da which confirmed the successful incorporation of hepatitis B polypeptide into ISCOMS(peptide failed to incorporate into ISCOMS was removed during dialysis). The peptide control showed a straight and regular band, while those for peptide-ISCOMS were broad which indicated effects of other components on SDS-PAGE separation. Specific CTL responses were markedly enhanced by ISCOMS-adjuvanted hepatitis B vaccine compared to antigen peptide control in mice visualized by ELISPOT assay. More work will be done to investigate the immunological properties and mechanisms underlying of this experimental ISCOMS-based vaccine from hepatitis B polypeptide *in vivo*.

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