Construction of Hybrid Gene of Hepatitis B Surface Antigen Carrying Heat-Stable Enterotoxin of *Escherichia coli* and Its Expression in Mammalian Cell Line

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ABSTRACT

Hepatitis B surface antigen is the first genetically engineered vaccine licensed for human use. Various strategies have been proposed to obtain a vaccine that would bypass the need for injection. In this study, a non-toxic portion of heat-stable enterotoxin of *Escherichia coli* that is capable of adhering to epithelial cells was inserted at amino acid position 112 of hepatitis surface antigen. The construct was used for transfection of human embryonic kidney cells in order to assess the expression of the hybrid protein. The data obtained showed a very low level of expression. *In vivo* antibody production and cytotoxic T lymphocyte response in B6 mice were assessed using DNA immunization. Three out of five injected mice responded with titers 010 mIU/ml anti-HBsAg and cytotoxic T-lymphocyte response was much higher with construct encoding the chimeric protein. Although this study proves that the chimeric protein is capable of eliciting both humoral and cellular responses, but further work is required to fully explore the feasibility of combining the properties of the two proteins. *Iran. Biomed. J. 6 (2 & 3): 47-53, 2002*

INTRODUCTION

From the early 1980s, interest in mucosal immunization has increased. The principal factors responsible for this revival relate to the technological advances, a better understanding of the mucosal immune regulation, cost of producing and delivering recombinant parenteral vaccines and better patient compliance [1]. Induction of immune responses at the mucosal surfaces is facilitated by delivery of the antigen into the epithelial cells at the mucosal surfaces. Bacterial toxins exhibit several properties that make them promising candidates such as entry into the mucosal cells and induction of both humoral and mucosal immune responses [2, 3].

Hepatitis B surface antigen (HBsAg) has been the first recombinant subunit vaccine produced for widespread use in humans. The primary structure of HBsAg can be divided into three hydrophobic and two hydrophilic regions. The aminoterminal part, contains two hydrophobic regions, signals I and II consisting of residues 11-29 and 80-98, respectively. Deletions in signal I renders HBsAg unable to take part in further assembly steps and deletion of signal II completely destabilizes the chain [4]. The carboxyterminal part between residue 217 and 226 has been reported to induce an antibody response in the immunized individuals [5]. Therefore, it is believed that this region resides outside the membrane in the native state, despite its clearly hydrophobic character. Deletion of the C-terminal domain produces a stable, glycosylated, but secretion-deficient mutant protein [6]. Sequence part 120-160 is external and forms the HBV-specific determinant 'a', which is common to all serological types [7]. The most important parts of this conformational determinant are between residues 121 and 147 [8]. Eight cysteines are located in the external region of HBsAg, but it is not known which of them form intra- or inter-molecular disulfide bridges [9]. However, serological studies show that the isolated peptide region 130-147 increases its serological affinity when it is cyclisied by an interchain disulfide bridge. Recent studies suggest that the structure of

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the external part is established by disulfide bonds between residues C124-C137 and C139-C147 [10].

HBsAg which is assembled by approximately 100 protein molecules, has been used as a particulate carrier for various foreign gene products [11]. The hybrid particles could present a large amount of the protein produced by the inserted sequence. It has been shown that insertion at amino acid position 113 of HBsAg appears to favor the expression of heterologous sequences [12, 13].

Mucosal immunity is difficult to achieve with subunit vaccines unless such vaccines are administered with an adjuvant. Bacterial toxins have been used as either adjuvant or in chimeric forms to incite and sustain an immune response against the desired antigen [2, 3, 14, 15]. *Escherichia coli* produces a low molecular weight toxin that has receptors on the epithelial cell of the intestine [16]. It has been shown that truncated form of this toxin is attenuated but retains its receptor binding ability [17]. Since intranasal administration of HBsAg alone has failed to induce an immune response, this study was undertaken in order to assess the feasibility of combining the features of both proteins. In this investigation, a vector containing the HBsAg with the insertion of the truncated form of heat-stable enterotoxin of *E. coli* at amino acid position 112 was constructed in order to assess the expression of the chimeric gene. The protein was expressed using human embryonic kidney cells and i*n vivo* antibody production and cytotoxic T lymphocyte response in C57BL/6J (B6) mice was assessed using DNA immunization.

MATERIALS AND METHODS

Synthetic gene for heat-stable enterotoxin. Truncated form of the gene for heat-stable enterotoxin of *Escherichia coli* was amplified using two overlapping oligonucleotides incorporating two *Bam*HI sites with the following sequences:

ST1 Forward primer; 5'- CGC<u>GGA TCC</u> GCT GTG AAC TTT GTT GTA ATC C-3'

ST2 Reverse primer; 5'- CGC<u>GGA TCC</u> ACA TCC TGT ACA GGC AGG CTT ACA-3'

PCR was performed for 25 cycles with Vent DNA polymerase (New England Biolab) Denaturation at 94°C for 1 min, annealing at 65°C for 45 s and polymerization at 72°C for 45 s. The 53 bp amplified product was visualized after electrophoresis in 15% polyacrylamide gel stained with ethidium bromide.

Construction and cloning of the chimeric gene. The amplified fragment encoding the truncated form of heat-stable enterotoxin of *E. coli* was digested with *Bam*HI and was used for in frame insertion into the S gene (subtype *ayw*) cloned in pLAS (obtained from Pasteur Institute of Paris). PLAS contains a *Bam*HI site at position 339 corresponding to amino acid 112-113, that led to duplication of amino acid 112-113 (Gly-Ser) and insertion of 13 amino-acid sequence between the duplicated residues (Fig. 1). The site of insertion into the HBsAg is part of a hydrophilic domain of the viral envelope protein and is closed to the region carrying most of the HBs antigenic determinants.
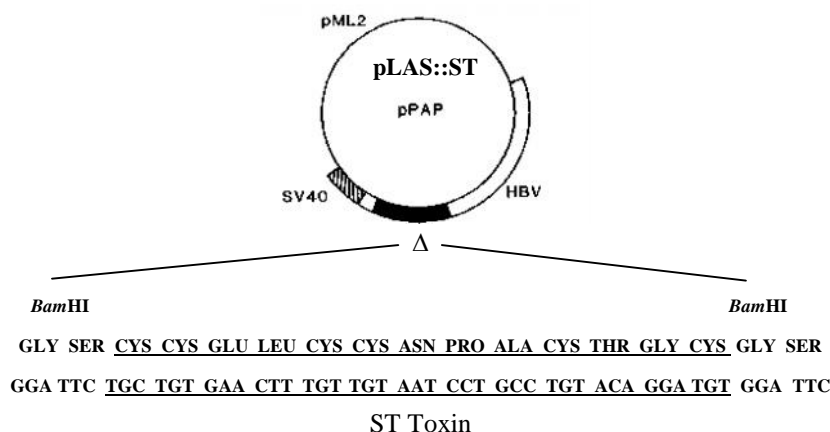

Fig. 1. pLAS::ST schematic map.

The orientation of ST gene was checked by PCR using HBS1 forward (5'-CCCT GCG CCATGG ATG GAG AAC ATC ACA TCA G-3') and ST2 reverse (5'- CGCGGA TCC GCT GTG AAC TTT GTT GTA ATC C-3') primers. The presence of a 399-bp PCR product indicated the write orientation. Sequencing was performed with ALF automated sequencing system (Pharmacia) using universal primers and verified by gatc Biotech co., Germany. The chimeric HBsAg::ST gene was subcloned in *p*CI/ *Eco*RI-*Not*I. To introduced *Eco*RI and *Not*I sites into the flanking regions of the HBsAg::ST encoding gene, this gene was amplified from the plasmid pLAS::ST using the primers; HBS1 Forward primer; 5'-CCCT GCG CCATGG ATG GAG AAC ATC ACA TCA G-3' and HBS2 Reverse primer; 5'-TTT TGT GCGGCCGC-TTA AAT GTA TAC CCA AAG AC-3'.

Amplification was performed for 30 cycles using *vent* DNA polymerase (New England BioLab) at 94°C denaturation for 1 min, 55°C annealing for 45 s and 72°C polymerization for 45 s. The 731-bp PCR product was visualized with UV after electrophoresis in 0.8% agarose stained with ethidium bromide.

The plasmid pCI (Promega) has a strong human cytomegalovirus (HCMV) intermediate early promoter and a ColE1 origin of replication. This vector is designed for the expression of recombinant proteins in mammalian cells and was used for expression of the chimeric HBsAg::ST in human embryonic kidney cell line and DNA immunization. A pCI vector encoding HBsAg alone was also constructed and used as control.

Transfection and expression. Human embryonic kidney cells (10^6) were transiently transfected with 10 µg of pCI/S-ST DNA using $CaPO_4$ method, and cultured for 2 days. The cells were then labeled with 100 µCi of $[^{35}S]$-methionine (Amersham, Braunschweig, Germany) in methionine-free RPMI-1640 medium. The labeled cells were washed twice in PBS and extracted with lysis buffer [120 mM NaCl, 1% v/v aprotinin (trasylol), 50 µM leupeptin, 0.5% v/v Nonidet P-40, 10% v/v glycerol, 50 mM Tris; HCl (pH 8.0)] at 4°C for 30 min. The extracts cleared by centrifugation at $20,000 \times g$ at 4°C for 30 min were subsequently incubated at 4°C for 2 h with protein-A Sepharose (Pharmacia, Freiburg, Germany). The HBsAg/ST protein was precipitated with a polyclonal rabbit anti-HBs antiserum and protein-A Sepharose at 4°C and washed extensively in 0.5 M LiCl. 1% v/v NP-40 and 0.1 M Tris HCl (pH 9.0).

After two additional washes in $1 \times$ PBS and two washes in $0.1 \times$ PBS the HBsAg-containing immune complexes were recovered from protein-A Sepharose in 400 µl elution buffer. After a 30-min incubation at 37°C, SDS denatured eluates were lyophilized and dissolved in 30 µl aqueous solution of 5% v/v 2-mercaptoethanol, 10% v/v glycerol and bromophenol blue, samples were analysed by 12.5% SDS-PAGE after boiling for 2 min. The gel was dried and visualized using X-ray film.

DNA immunization of mice. DNA inocula were prepared by anion exchange column (Maxi Prep, Qiagen, Germany) from each plasmid preparation. DNA was resuspended in endotoxin-free sterile physiological saline and used to immunize female C57BL/6J (B6) mice bred under SPF conditions in the animal colony of Ulm University, Ulm, Germany. Three groups of five animals were injected according to the protocols of Schirmbeck *et al*. [18]. Briefly, mice were first anaesthetized with sodium pentobarbital and then 100 µg of plasmid DNA was injected into the tibial anterior muscles of the hind legs (50 µg in each leg). The muscle was pretreated 5 days before the DNA injection by an intramuscular injection of 50 µl of 0.01 mM cardiotoxin (Latoxan, Rosans, France). Mice were boosted with the same dose of plasmid DNA, 4 weeks after the first injection, and sera were collected 3 and 7 weeks after the first inoculation.

Determination of serum antibody levels. Serum samples were obtained from individual mice by tail bleedings 3 and 7 weeks post-injection. Antibodies against HBsAg were detected in mouse sera with the commercially available test IM × AUSAB (Abbott, Wiesbaden, Germany). In this assay, microparticles coated with HBsAg were mixed with mouse serum samples to allow specific binding of anti-HBsAg antibodies. Microparticles coated with antigen-antibody complex were transferred and irreversibly bound to a glass fiber matrix. After the addition of biotinylated recombinant HBsAg, binding to the complex was traced by an alkaline phosphatase conjugated antibiotic antibody followed by the addition of the substrate 4-methyl-lumbelliferyl-phosphate. Antibody levels were quantified using six standard sera (0-1000 mIU/ml). Values presented are calculated by multiplying the serum dilution with the measured antibody level (mIU/ml).

Cytotoxic assay. Single cell suspensions prepared

from spleens of immunized mice were suspended in a MEM tissue culture medium supplemented with 10 mM HEPES buffer. 5×10^{-5} M 2-ME, antibiotics and 10% v/v FCS (Pan Systems. Aidenhach, Germany). Responder cells (3×10^7) were co-cultured with 1.5×10^6 syngeneic, irradiated (20,000 rad) RBL5/S or P815/S transfectants in 10 ml medium in a humidified atmosphere and 7% CO_2 at 37°C. After a 5-day co-culture, blast cells were harvested, washed and re-stimulated for a further 4 days. After the 9 days *in vitro* re-stimulation culture, serial dilutions of effector cells were cultured with 2×10^3 ^{51}Cr-labelled targets in 200 μl round bottom wells. Specific cytolytic activity of the cells was tested in 4h ^{51}Cr-release assays against peptide pulsed or transfected target cells and compared with non-pulsed and non-transfected controls. After a 4-h incubation at 37°C 100 μ1 of supernatant fluid was collected for γ-radiation counting. The percentage specific release was calculated as [(experimental release—Spontaneous release)/(total release—spontaneous release)] × 100. Total counts were measured by resuspending target cells. Spontaneously released counts were always less than 15% of the total counts. Data shown are the mean of triplicate cultures. The standard error of the mean (S.E.M.) of triplicate data was always less than 15% of the mean [18].

RESULTS

A 13 amino acid long truncated form of *Escherichia coli* heat-stable (hSTa) that is protease-resistant and methanol-soluble was inserted at amino acid position 112 of HBsAg and cloned in eukaryotic cell expression vector (Fig. 2). The construct pCI/S::ST was transfected into human embryonic kidney cells and cellular clones were screened for HBsAg expression by radioimmunoprecipitation. The expression of both glycosylated and non-glycosylated forms of the chimeric protein was observed in the cell lysate, but the expression level was low and the secretary form of the protein could not be detected (Fig. 3). The difference between the apparent molecular weight of the HBsAg and the chimeric protein was in good agreement with the amino acid sequence encoded by the insert.

Protection against HBV infection is mediated by antibodies to HBsAg. In order to evaluate the immunogenic properties of the HBsAg/ST protein,

B6 mice were inoculated with constructs expressing the hybrid protein and HBsAg alone. The injection of mice with construct expressing native HBsAg gave high, but variable results and variation was also observed when mice were injected with the plasmid constructed to express mixed particles of HBsAg and HBs/ST. Mice immunized with pCI/S::ST responded only weakly (>10 mIU/ml) even after second boost (mice 1, 2 and 3) and in two mice (4 and 5) the antibody response was negative after second boost (Table 1).

Cytotoxic T lymphocyte activity appears to play an important role in resolving HBV infection; therefore, CTL response of the hybrid protein was also assessed in immunized mice. As it is shown in Figure 4, the HBsAg-specific CTL response is higher with the plasmid encoding the chimeric HBsAg-ST than the vector encoding native HBsAg.
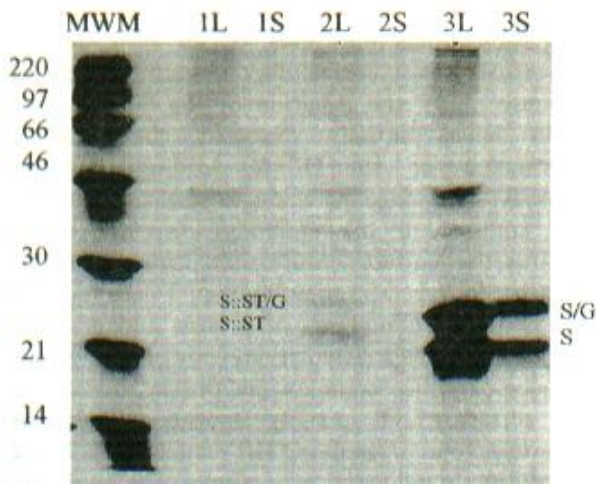
(A)
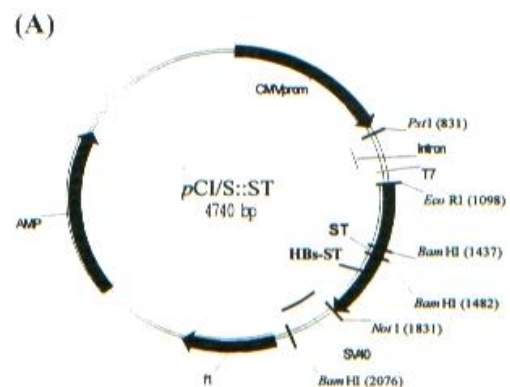
(B)

PCI/S::ST   112   113

GLY   SER   <u>CYS   CYS   GLU   LEU   CYS   CYS</u>

GGA   TTC   TGC   TGT   GAA   CTT   TGT   TGT

                                    114

<u>ASN   PRO   ALA   CYS   THR   GLY   CYS</u>   GLY

<u>AAT   CCT   GCC   TGT   ACA   GGA   TGT</u>   GGA

Fig. 2. pCI/S::ST schematic map. **(A)**, Plasmid pCI/S::ST carrying the chimeric HBsAg::ST; **(B)**, structure of chimeric gene showing amino acid sequence of the truncated ST inserted in HBsAg coding region.

Fig. 3. SDS-PAGE analysis of HBsAg and hybrid HBsAg::ST proteins. [^{35}S] methionine-labeled proteins from cellular lysates (L) and supernatants (S) were immunopercipitated with antiserum to HBsAg. Lanes 1L and 1S, pCI (Negative control); Lanes 2L and 2S, pCI/S::ST; Lanes 3L and 3S, pCI/S (positive control). S/G, (S Ag Glycosylated; S, S Ag non-glycosylated; S::ST/G, S::ST Ag Glycosylated; S::ST, S ST Ag non-glycosylated.

DISCUSSION

A truncated form of the gene encoding a heat-stable enterotoxin of *Escherichia coli* was inserted into the second hydrophilic region in HBsAg corresponding to amino acid 112. The expression of the modified gene was assessed in human embryonic kidney cells, which expressed hybrid HBsAg protein in both glycosylated and non-glycosylated forms. However, the chimeric protein could not be detected in cellular supernatants (Fig. 3) and could only be immunoprecipitated from lysate of the corresponding clones.

How the insertion of ST-encoding fragment had impaired secretion remains speculative. Delpeyroux *et al.* [19] showed that several factors including the hydrophobicity of the inserted sequence and prevention of the formation of appropriate disulfide bridges can affect the secretion level of the chimeric proteins.

Of the 14 cysteines present in the HBV envelope protein, eight are located in the second hydrophilic loop. These residues are highly conserved among the mammalian hepadenoviruses and HBV subtypes [20, 21]. Intra-and inter-molecular disulfide bonds among these residues contribute to the compact, protease-resistance structure of the particles. Mutational analysis has revealed that the eight cysteines are not equally important [9]. Mutations involving cysteines 121 and/or 124 are efficiently secreted and the double mutant Cys147/149Ala is secreted with intermediate efficiency. However, interaction with cellular chaperones because of an incorrect folding causing the block to secretion has been put forward as an explanation for the secretion-defective phenotypes [22].
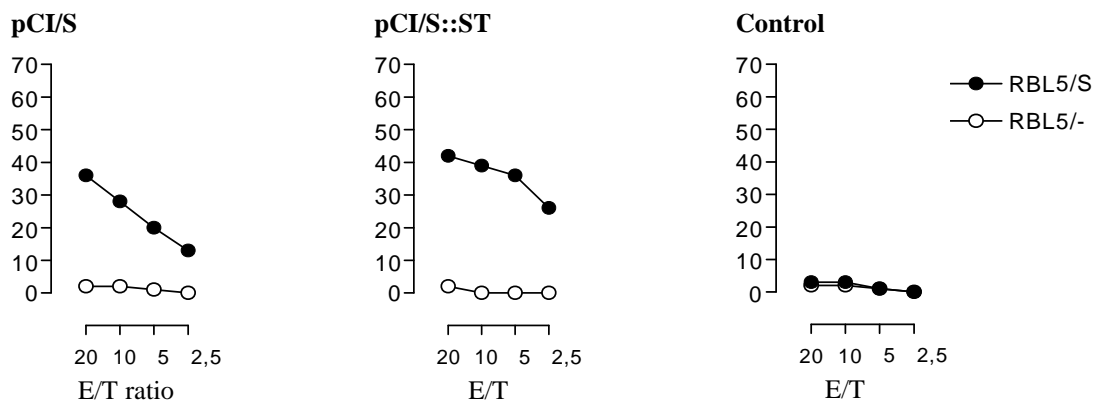
Fig. 4. Priming CTL to HBsAg by injecting different plasmid DNA. Mice were immunized with 100 µg of pCI/S, pCI/S::ST i.m. (m. tiabilis anterior). Ten days after DNA injection, spleen cells from immunized mice were re-stimulated *in vitro* with RBL/S, RBL5/- transfectants. The specific cytolytic reactivity of primed CTL was tested against P815/S targets in a 4 h ^{51}Cr release assay. Controls are spleen cells from non-immunized mice. Plots are means specific lysis value from individual, representative mice tested at the indicated effector/target (E/T) ratio.

The inserted fragment of ST contains 6 cystein residues and introduction of this cystein-rich fragment could possibly have interfered with the inter- and intra- disulfide bond formation required for correct folding of the HBs protein. This in turn could result in our inability to detect the hybrid protein in the supernatant of the transfected cells. The misfolding of the expressed hybrid protein could also be a reason for low expression of this protein compared with the expression level of HBsAg using the same construct (Fig. 3)

The mice immunized with the construct expressing the chimeric HBsAg::ST responded with anti-HBsAg antibody titers of >10 m IU/ml, which is sufficient for protection against vial infection [23]. However, the level of antibody production was about 15-400 times lower than the antibody titer obtained with vector expressing HBsAg alone. The reason for this discrepancy was not ascertained, but could be due to the difference in expression level of the two proteins and/or the fact that the chimeric HBs::ST is not secreted as was shown *in vitro* cell culture assay (Fig. 3). Mancini *et al.* [24] reported a lack of antibody production to L protein of HBV and concluded that the lack of antibody production was due to the fact that myogenic cells did not secrete these particles. Bryder *et al.* [25] also found that elimination of secretion by single amino-acid mutation in the HBsAg decreased the anti-HBsAg antibody response. Furthermore, in view of the suggestion that the level of *in vivo* antigen expression is not critical for triggering a humoral response [26], the insertion of the ST fragment might have partially distorted the antigenic determinant of the HBsAg leading to lower antibody response to the chimeric particle.

In contrast to the antibody response, which requires the correct conformational structure of the antigen, the specific stimulation of T cells requires the partial degradation of protein antigens into peptides and their association with class I major histocompatibility complex and presentation on the cell surface. CTL activity appears to play an important role in resolving HBV infection [27] and the ability to induce such responses is an important goal for developing effective therapeutic and preventative vaccines. In this study, the CTL response to the HBsAg was improved with the construct expressing chimeric HBsAg::ST. This result and production of HBsAg specific antibody after injection of mice with the pCI/S-ST confirm the proper *in vivo* expression of the hybrid protein. However, further work, is required to improve the expression of this chimeric protein, in order to ascertain the receptor-binding ability of the hybrid protein and also to improve the antibody response to HBsAg.

Table 1. Anti-HBsAg response in mice immunized with different constructs. The serum antibody response of B6 mice immunized with mono- and di-cistronic (bi-directional) plasmid DNA. B6 mice were immunized by i.m. injection of 100 μg DNA: pCI/S (encoding HBsAg), pCI/S::ST (encoding HBs::ST) and di-cistronic pBI/S-S::ST (encoding HBsAg and HBs::ST). Mean titers of anti-HBs antibodies in sera of the mice obtained 3 weeks post-immunization. The cut-off point of this assay is 10 ml U/ml.

Construct	Mice (no)	Anti-HBs titer (m IU/ml)	
		After 1ST injection	After 2^{ed} injection
PCI/S	1	35	339
	2	682	8867
	3	64	1130
	4	3	342
	5	1265	3042
PCI/S::ST	6	1.5	18.0
	7	1.2	22.0
	8	0.3	38.0
	9	1.5	7.0
	10	1.2	7.5
PBI/S-S::ST	11	414	5641
	12	13.7	1320
	13	0.1	25

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