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High affinity mouse-human chimeric Fab against Hepatitis B surface antigen

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Abstract

AIM: Passive immunotherapy using antibody against hepatitis B surface antigen (HBsAg) has been advocated in certain cases of Hepatitis B infection. We had earlier reported on the cloning and expression of a high affinity scFv derived from a mouse monoclonal (5S) against HBsAg. However this mouse antibody cannot be used for therapeutic purposes as it may elicit anti-mouse immune responses. Chimerization by replacing mouse constant domains with human ones can reduce the immunogenicity of this antibody.

METHODS: We cloned the V_H and V_L genes of this mouse antibody, and fused them with CH1 domain of human IgG1 and C_L domain of human kappa chain respectively. These chimeric genes were cloned into a phagemid vector. After initial screening using the phage display system, the chimeric Fab was expressed in soluble form in *E. coli*.

RESULTS: The chimeric Fab was purified from the bacterial periplasmic extract. We characterized the chimeric Fab using several *in vitro* techniques and it was observed that the chimeric molecule retained the high affinity and specificity of the original mouse monoclonal. This chimeric antibody fragment was further expressed in different strains of *E. coli* to increase the yield.

CONCLUSION: We have generated a mouse-human chimeric Fab against HBsAg without any significant loss in binding and epitope specificity. This chimeric Fab fragment can be further modified to generate a full-length chimeric antibody for therapeutic uses.

Key words: Chimeric Fab; Hepatitis B surface antigen; Phage display

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INTRODUCTION

Hepatitis B virus (HBV) infection is the 10th leading cause of death worldwide, with 2 billion people infected by it and 350 million suffering from chronic HBV infection[1]. Protective antibodies that appear after natural infection are mostly directed against the major antigenic 'a'determinant of Hepatitis B surface antigen (HBsAg)[2]. The immunodominant 'a' epitope is a part of a large antigenic area of HBsAg, called the major hydrophilic region[3] and this epitope is present in all serotypes[4]. Antibodies against HBsAg are thus advocated for passive immunotherapy against Hepatitis B infection in cases of accidental needle stick injuries, for liver transplant patients and to prevent vertical transfer of HBV infection from mother to child[5-8]. Presently, human anti-HBs immune globulin (HBIG) collected from the blood of hyperimmune donors is used for postexposure prophylaxis. Being a blood derived product anti-HBs HBIG is costly and can cause cross-contamination. Therefore a recombinant antibody to HBsAg can be a suitable alternative to such a practice.

Although several recombinant antibodies against HBsAg have been reported in literature, none is available for clinical use[9–12]. In our previous work we had expressed and characterized a recombinant anti-HBs scFv cloned from a mouse monoclonal 5S[13]. This antibody binds to HBsAg with high affinity (K_D = 0.889 nmol/L). It tested positive in an *in vitro* surrogate test for seroconversion and protective antibodies (Hepanostika anti-HBs kit, Organon Teknika, The Netherlands). The scFv generated from this hybridoma retained the high affinity and epitope specificity[13]. However this mouse monoclonal cannot be used for therapeutic purposes as it may trigger human anti-mouse antibody response, especially when multiple infusions are required to obtain therapeutic efficacy[14,15]. It is well known that immunogenic reactions are predominantly directed towards constant domains of

Table 1 Oligonucleotide primers used for generation of the chimeric Fd and light chain

Primer	Sequence
5′ primer for V_H (5H23M)	5′- AG GTC CAG CTT CTC GAG CCC GGG GC -3′
3′ primer for V_H (Fd3)	5′-CGA TGG GCC CTT GGT GGA GGC TGA AGA GAC AGT GAC TGA GGT TCC-3′
5′ primer for C_H1 of human IgG1 (Fd5)	5′-GGA ACC TCA GTC ACT GTC TCT TCA GCC TCC ACC AAG GGC CCA TCG-3′
3′ primer for C_H1 of human IgG1 (CG1Z)	5′- GCA TGT ACT AGT TTT GTC ACA AGA TTT GGG -3′
5′ primer for V_L (5L35)	5′-CCA GAT GTG AGC TCG TGA TGA CCC AGA CTC CA-3′
3′ primer for V_L (K3)	5′-CAG ATG GTG CAG CCA CAG TCC GTT TGA GTT CCA GCT TGG-3′
5′ primer for C_L of human κ chain (K5)	5′-CCA AGC TGG AAC TCA AAC GGA CTG TGG CTG CAC CAT CTG-3′
3′ primer for C_L of human κ chain (Ck1d)	5′- GCG CCG TCT AGA ATT AAC ACT CTC CCC TGT TGA AGC TCT TTG TGA CGG GCG AAC TCA G -3′

Primer names are given in parenthesis. Sites for restriction enzymes are shown as underlined. Complementary overhangs are shown in bold letters.

murine antibodies[16]. Problems associated with the HAMA response can be reduced by creating mouse-human chimeric antibodies, where mouse constant regions are replaced by human ones[17-19].

The exact mechanisms of antibody-mediated virus neutralization are not clear till date. A few of the principal mechanisms, which have been postulated for virus neutralization, are virus aggregation, inhibition of attachment of virus to cell receptors and inhibition of events after attachment to cell receptors[20,21]. Though high affinity binding to viral epitopes is a pre-requisite for antibody-mediated virus neutralization, the importance of antibody constant domains is not clear. Apart from full-length antibodies, antibody Fab fragments have been shown to neutralize viruses[22-26]. It has been shown that $F(ab)_2$ fragments derived from HBIG is effective for the prevention of vertical transfer of HBV infection in neonates[27]. In comparison to a full-length antibody, a Fab fragment can be easily expressed in bacterial expression systems[28]. A recombinant Fab can be further modified for increase in affinity[29], for chimerization/humanization[30] and can be linked with antibody Fc region to generate a full-length antibody[31,32].

In the present work, we have fused the variable regions of the mouse monoclonal 5S (IgG1/κ) with the corresponding human constant regions (C_H1 of IgG1/C_L of κ) to generate a mouse-human chimeric Fab. This chimeric Fab was expressed using a phage display expression system. After initial screening of functional clones, the chimeric Fab was expressed in *E. coli* in soluble form. It was purified by affinity chromatography and characterized for antigen binding. We observed that the chimeric Fab fragment retained the high affinity binding and epitope specificity of the original mouse monoclonal. This chimeric molecule can be further modified for generation of a therapeutically useful full-length chimeric antibody.

MATERIALS AND METHODS

Materials

The phagemid vector pCOMB3H was provided by The Scripps Research Institute, La Jolla, USA. Shanta Biotech (India) provided purified recombinant HBsAg expressed in a *Pichia* system. *E. coli* XL1-Blue and TG1 cells and helper phage M13 KO7 were obtained from MRC, Cambridge, UK. *E. coli* strains AD494 and BL 21 CodonPlus were procured from Novagen. 5S hybridoma cells were maintained in RPMI (Sigma, USA) with 10% FCS (Sigma, USA). Anti-M13 mouse antibody was provided by Dr. Vijay Chaudhary, University of Delhi, South Campus, New Delhi, India.

Construction of the chimeric light chain and Fd fragment

The strategy for generation of the chimeric Fd and light chain is shown in Figure 1. The variable region genes of 5S hybridoma were amplified by reverse transcription followed by PCR. Primers used for all reverse transcriptions and PCRs are listed in Table 1. Total RNA was isolated from 5S cells using TRI reagent (Sigma, USA) and cDNAs for the V_H and V_L fragments were generated by reverse transcription using Omniscript RTase (Qiagen). Primers used for reverse transcriptions were Fd3 and K3 for V_H and V_L respectively. These primers have overhangs complementary to the 5' regions of the respective human constant domains. The V_H fragment was further amplified by PCR using primers 5H23M and Fd3. Similarly, the V_L fragment was amplified by PCR using primers 5L35 and K3. Conditions for both the PCRs were: 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, followed by a final extension for 10 min at 72 °C.

For amplification of human constant domains, RNA was isolated from human peripheral blood lymphocytes (PBLs) using TRI reagent. cDNAs for the C_H1 region of human IgG1 and the C_L region of human kappa chain were generated by reverse transcription using Omniscript RTase (Qiagen). Primers used for reverse transcriptions were CG1Z and Ck1d for C_H1 and C_L respectively. The C_H1 fragment was further amplified by PCR using primers Fd5 and CG1Z. Human C_L was amplified by PCR using primers K5 and Ck1d. Fd5 and K5 carry overhangs complementary to the 3' end of variable regions of 5S. Conditions for both these PCRs were: 30 cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min, followed by a final extension for 10 min at 72 °C.

PCR amplified fragments were resolved in 1.5% agarose gel and respective bands were eluted out using QIAquick gel extraction kit (Qiagen). PCR amplified C_H1 (human IgG1) and mouse V_H were used as templates for generation of the chimeric Fd by overlap PCR. Initial assembly of equimolar amounts of the mouse V_H and human C_H1 was done by PCR for 20 cycles at 94 °C for

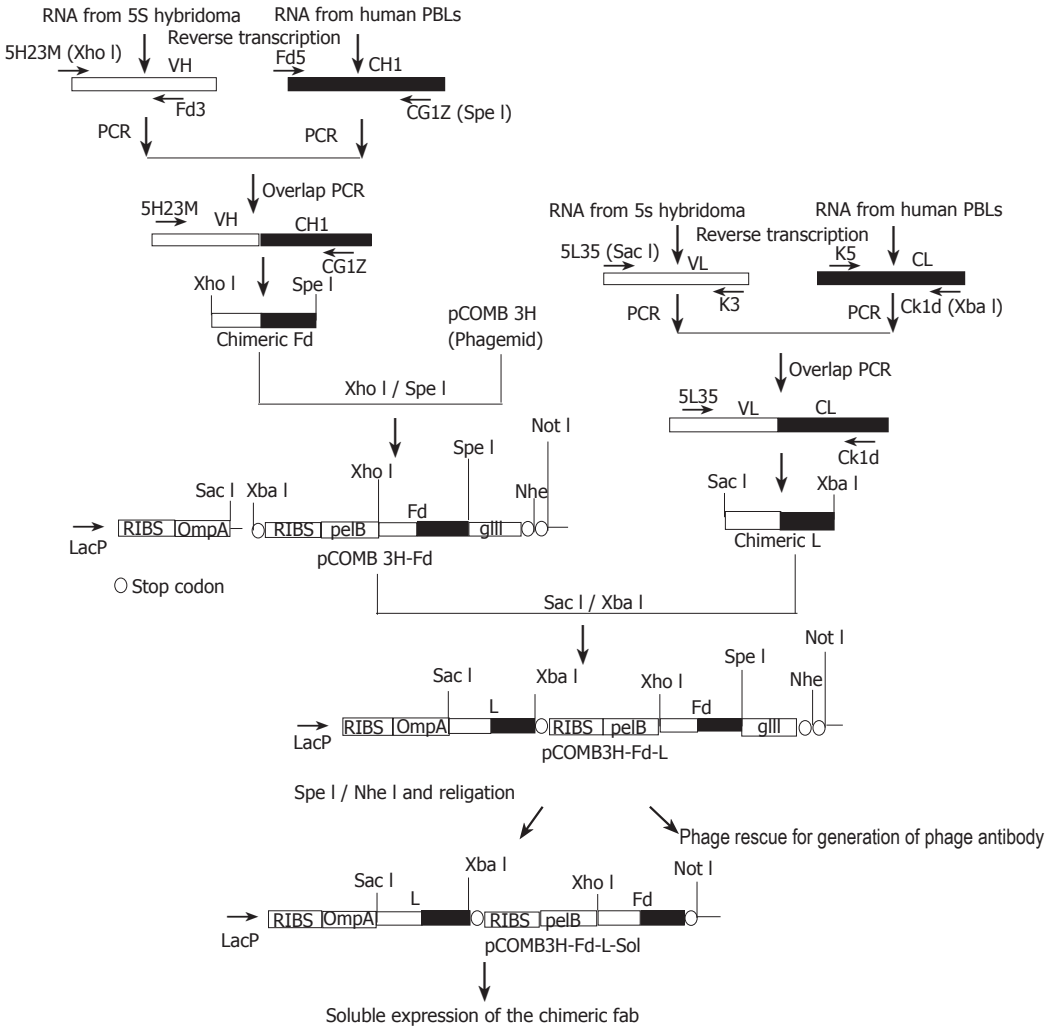

Figure 1 Schematic representation of the strategy for cloning and expression of the anti-HBs chimeric Fab. The V_H and V_L fragments of 5S hybridoma were fused with human C_H1 and C_L by overlap PCR. The resulting chimeric fragments were cloned in the bi-cistronic phagemid vector pCOMB3H. Both the fragments are under the control of a single *LacZ* promoter. *ompA* and *pelB* are two leader sequences, provided for directing the light chain and Fd fragment to the bacterial periplasm. Two ribosome-binding sites are present in the vector for stable expression of both the fragments. The Fd fragment was cloned upstream to the phage gIII sequence, for surface display of the chimeric Fab. This *gIII* fragment was removed by digestion with *Spe I/Nhe I* and after self-ligation of the vector, the Fab fragment was expressed in soluble form.

1 min, 60 °C for 1 min and 72 °C for 2 min, followed by final extension of 10 min at 72 °C. The product of the initial assembly reaction was diluted 10 times and used as the template for pull through PCR using primers 5H23M and CG1Z. Similarly human kappa C_L and mouse V_L fragment were joined to generate the chimeric light chain. Primers used for this reaction were 5L35 and Ck1d.

Construction of the phagemid construct

We used the bi-cistronic phagemid vector pCOMB3H, a variant of the phagemid pComb3[33], for the expression of the chimeric Fab. The essential features of this vector system and our cloning strategy are shown in Figure 1. The chimeric Fd was digested with *Xho1* and *Spe1* and cloned into the phagemid vector pCOMB3H. The resulting construct (pCOMB3H-Fd) was transformed into chemically competent *E. coli* XL1-Blue cells by standard chemical method ($CaCl_2$/heat shock)[34]. Transformed cells were grown on Amp-Agar plates. Colonies were picked up after overnight incubation and screened for the presence of the insert by colony PCR and restriction digestion (*Xho1*/*Spe1*). The recombinant phagemid pCOMB3H-Fd was isolated by alkali-lysis method and digested with *Sac1* and *Xba1*. The chimeric light chain digested with these two enzymes was cloned into pCOMB3H-Fd to generate the phagemid construct pCOMB3H-Fd-L. This construct was transformed into chemically competent XL1-Blue cells by standard chemical transformation method and after overnight incubation, recombinant clones were checked for the presence of the chimeric light chain by colony PCR and by restriction digestion (*Sac1*/*Xba1*).

Expression of the phage antibody and selection of antigen binding clones

Cells transformed with the construct pCOMB3H-Fd-L were used for expression of the chimeric Fab on phage surface. Phage displaying chimeric Fab was rescued by infection with helper phage M13-KO7 as described by

Barbas *et al*.[33]. Phage was precipitated from the culture supernatant by incubating with PEG/NaCl (20% PEG/2.5 mol/L NaCl) for 8 h on ice. After centrifugation, precipitated phage was resuspended in PBS and titrated to determine phage concentration[35].

Clones displaying functional Fab fragment were selected by biopanning over antigen-coated plates. The method for biopanning has been discussed in detail in our earlier article[13]. Essentially this method involves incubation of phage in uncoated ELISA plate followed by incubation of unbound phage in antigen-coated plate. After thorough washing, bound phage was eluted out at low pH. Eluted phage was passed through two more rounds of selection over coated and uncoated wells with increasing number of washing.

Identification of antigen binding clones by phage-ELISA

Phage obtained after three rounds of selection, was reinfected in XL1-Blue cells and amplified by standard phage rescue protocol as mentioned earlier. Antigen binding clones were identified by phage-ELISA. Maxisorp ELISA plates were coated with HBsAg (250 ng/well) in bicarbonate buffer (pH 9.5). After blocking with 2% non-fat milk in PBS (MPBS), phage (~10^{12}/well) was added and incubated for 1 h at room temperature. Bound phage was detected by incubation with 1:1 000 dilution of anti-M13 mouse antibody for 1 h at room temperature, followed by 1 h incubation with 1:2 000 dilution of anti-mouse antibody-HRP conjugate (Promega, USA). ELISA was developed by using 100 μL of 0.4 mg/mL *o*-phenylenediamine and 0.8 μL/mL of H_2O_2 in citrate-phosphate buffer (pH 5). The same amount of helper phage (M13-KO7) was used as negative control in phage-ELISA.

The phagemid construct was isolated from the clone showing maximum binding and the nucleotide sequences of the chimeric Fd and light chain were determined by sequencing using ABI-Prism automatic DNA sequencer. Primers used for sequencing the light chain were 5'-ATGAAAAAGACAGCTATCGC-3' and 5'-TAATAAC AATCCAGCGGCTG-3'. Primers used for sequencing the Fd fragment were 5'-TCTTTTCATAATCAAAATCACC G-3' and 5'-AAATGAAATACCTATTGCC-3'.

Soluble expression of the chimeric Fab

The antigen binding clone selected by phage ELISA was further processed for soluble expression of the anti-HBs chimeric Fab. For soluble expression of the chimeric Fab, the phage *gIII* sequence was removed from the recombinant phagemid construct pCOMB3H-Fd-L by double digestion with *Spe I* and *Nhe I*. Digestion by these two enzymes provides compatible ends for self-ligation of the vector. Double digested phagemid was self-ligated to generate the construct pCOMB3H-Fd-L-Sol and transformed in chemically competent XL1-Blue cells. For soluble expression of recombinant chimeric Fab, 1 L Super Broth with 20 mmol/L $MgCl_2$ and ampicillin (100 μg/mL) was inoculated with 10 mL of overnight culture of the recombinant clone and grown at 37 °C till the A_{600} reached approximately 0.6, when overexpression of the chimeric Fab was induced by 1 mmol/L IPTG. After overnight growth at 30 °C, cells were harvested by centrifugation at 4 000 r/min. Cell pellet was re-suspended in 20 mL PBS/ 1 mmol/L EDTA and kept on ice for 40 min. Clarified periplasmic extract was obtained by centrifugation of the re-suspended product at 10 000 *g*. The same method was used to express the soluble chimeric Fab in three other *E. coli* strains (AD494, BL21 CodonPlus and TG1).

Purification of the recombinant chimeric Fab

The periplasmic extract was concentrated (~10 times) using Centriprep YM-30 centrifugal filter (Millipore, USA). The recombinant chimeric Fab was purified from the periplasmic extract by affinity chromatography using HiTrap Protein G HP column (Amersham) as per the manufacturer's protocol. In brief, the protein G column (1 mL) was washed thoroughly with double distilled water (five column volumes) and equilibrated with five column volumes of equilibration buffer (pH 7.0). The concentrated periplasmic extract was allowed to pass through the column using a syringe at a speed of 2 mL/min. The column was washed thoroughly by 10 volume of equilibration buffer and bound chimeric Fab was eluted out by 5 volume of elution buffer (pH 2.7). Eluted fractions were immediately neutralized using neutralization buffer (77 μL/mL, pH 9). The eluted fractions were concentrated by Centriprep YM-30 centrifugal filter and checked on SDS-PAGE.

Electrophoresis and Western blot analysis

The purified chimeric Fab was resolved by 12% SDS-PAGE, separately in reducing (with βME) and non-reducing conditions (without βME or DTT). The resolved protein was stained by silver staining.

For Western blot analysis, the concentrated periplasmic extract was resolved by 12% SDS-PAGE in non-reducing conditions and electroblotted on nitrocellulose membrane. After blocking with 4% MPBS for 2 h, the chimeric Fab was detected by Rabbit anti-Human IgG-HRP conjugate (1:1 000 dilution; Dako). The blot was developed using DAB/H_2O_2 system. An equal amount of concentrated periplasmic extract of untransformed XL1-Blue cells was used as the negative control for the Western blot experiment. A separate Western blot experiment was performed under reducing conditions for the detection of the monomeric Fd and light chain. Whenever required, intensity of bands in Western blots was measured densitometrically using ChemiImager 4400 (Alpha Innotech Corp., USA).

Antigen binding assays for the chimeric Fab

Binding of the chimeric Fab was checked by solid phase ELISA. Maxisorp ELISA plate was coated with 250 ng/well of HBsAg in bicarbonate buffer (pH 9.5). After blocking with 4% MPBS, different dilutions of the chimeric Fab was added to antigen coated wells and bound antibodies were detected by Rabbit anti-Human IgG-HRP conjugate (1:2 000 dilution; Dako). Hybridoma supernatant of the original mouse monoclonal 5S was

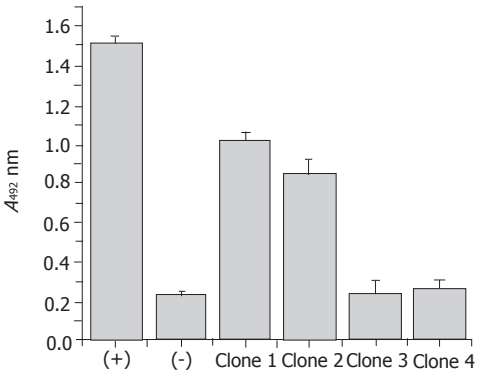

Figure 2 Phage ELISA to identify antigen binding clones. After three rounds of biopanning, selected phage was rescued and antigen-binding clones were detected by phage ELISA. In this experiment 5S hybridoma supernatant and helper phage M13 KO7 were used as positive (+) and negative (–) control respectively. Clone 1 was used for soluble expression of the chimeric Fab.

used as a positive control for this experiment. The extract of untransformed XL1-Blue cells was used as a negative control and corresponding readings were deducted from the readings of the chimeric Fab. This ELISA method was also used to study the levels of expression the chimeric Fab in different *E. coli* strains.

For competitive ELISA, plates were coated with HBsAg (100 ng/well) in bicarbonate buffer and blocked with 4% MPBS. Different amounts of the chimeric Fab were mixed with 1:80 dilution of 5S hybridoma supernatant and added to antigen-coated wells. After one hour incubation at room temperature, bound mouse antibody was detected by anti-mouse-HRP conjugate (Bangalore Genei, India). Periplasmic extract of untransformed XL1-Blue cells was used as the negative control and binding of the mouse monoclonal in its presence was taken as the maximum binding to calculate the percent inhibition.

Determination of the dissociation constant of the chimeric Fab

The dissociation constant (K_D) of HBsAg and chimeric Fab interaction was determined in solution phase by an ELISA method as described by Friguet *et al*.[36]. Essentially the technique involves incubation of a fixed amount of the antibody with different amounts of the antigen in solution phase for a prolonged period so that the equilibrium is reached. This is followed by detection of unbound antibody by conventional ELISA. This ELISA data is then used to calculate the K_D value, by using an equation derived from the law of mass action. The advantage of this technique is that it can be used to determine the affinity of an antibody without prior purification.

For this experiment we used crude periplasmic extract of the clone expressing the chimeric Fab in soluble form. A fixed amount of the periplasmic extract (1:300 dilution, in 100 μL of 4% MPBS) was incubated with varying concentrations of HBsAg (3-10 nmol/L) for 16 h at 4 °C. This equilibrated solution was incubated in antigen coated ELISA plates (250 ng antigen/well) for 20 min at room temperature to capture the free Fab. Bound chimeric Fab was detected by Rabbit anti-human IgG-HRP conjugate (Dako).

Protein estimation

Protein concentrations were estimated by Bradford assay[37].

RESULTS

Generation of a phagemid construct for the expression of phage antibody: Variable regions of the light chain and heavy chain of the mouse monoclonal 5S were amplified by RT-PCR. The $C_{H}1$ region of human IgG1 and C_L of human kappa chain were amplified by RT-PCR using RNA extracted from human PBLs. After the fusion of the mouse V_H and human $C_{H}1$, resulting chimeric Fd was cloned into the phagemid vector pCOMB3H to generate the recombinant construct pCOMB3H-Fd. Similarly, the chimeric light chain, generated by joining the mouse V_L and human C_L, was cloned into pCOMB3H-Fd to generate the recombinant construct pCOMB3H-Fd-L. This construct was transformed in XL1-Blue cells and phage displaying the chimeric Fab was generated by phage rescue using helper phage.

Selection of antigen binding clones and phage ELISA: The anti-HBs chimeric Fab was first expressed as phage antibody and antigen-binding clones were enriched by bio-panning over antigen coated plate. Selection was done on uncoated and antigen coated wells alternatively, thereby removing plastic binding clones, which can interfere in subsequent ELISAs. After three rounds of selection, functional clones were amplified and rescued to generate phage particles displaying the chimeric Fab and antigen-binding clones were detected by phage ELISA (Figure 2). As shown in Figure 2, clone 1 had the maximum binding and was used for soluble expression of the chimeric Fab. Nucleotide sequences of the chimeric Fd and light chain of this clone were submitted to the EMBL Nucleotide Sequence Database (EMBL accession numbers: AJ878860 and AJ878861). Sequence analysis indicated that the variable regions in these chimeric genes are identical to the variable regions of the mouse monoclonal 5S (data not shown).

Soluble expression of the chimeric Fab: Phage *gIII* sequence was removed from pCOMB3H-Fd-L for soluble expression of the chimeric Fab. The anti-HBs chimeric Fab was expressed in *E. coli* XL1-Blue cells by inducing overnight with 1 mmol/L IPTG. The leader sequences, present upstream to the chimeric genes, drag both the chains to the bacterial periplasm, where they form inter- and intra-chain disulphide bonds. The periplasmic extract was concentrated and the chimeric Fab was purified using Protein G column. Yield of the purified chimeric Fab when expressed in XL1-Blue cells was ~40 μg/L of culture. The purified product was resolved by SDS-PAGE in non-reducing (without βME/DTT) as well as in reducing conditions (with βME). As shown in Figure 3A, the chimeric Fab is expressed as a heterodimer (~50 ku) of the chimeric Fd and light chain. In reducing conditions, both the chains were detected in monomeric form (~25 ku).

Expression of the heterodimeric chimeric Fab was further confirmed by Western blot in non-reducing

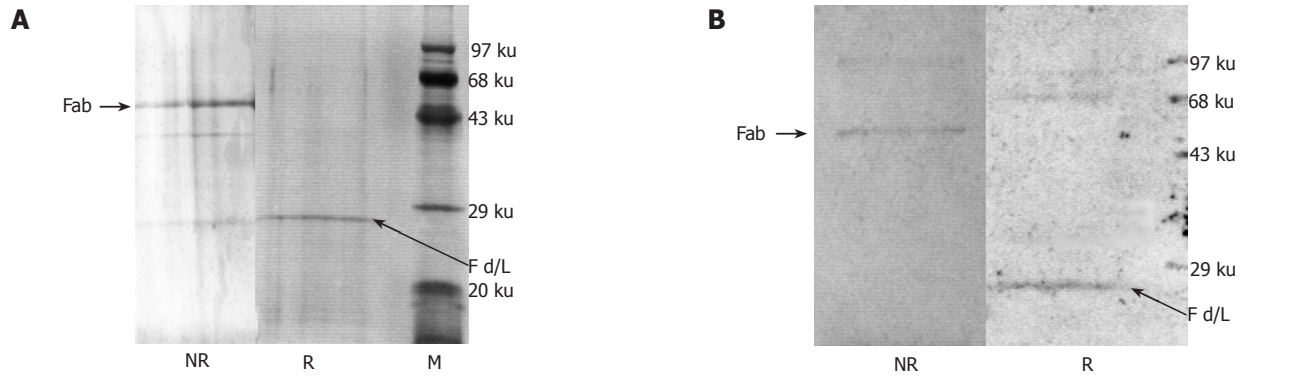

Figure 3 SDS-PAGE (**A**) and Western Blot (**B**) analysis of the chimeric Fab. The purified chimeric Fab was resolved in 12% SDS-PAGE under reducing (R) and non-reducing conditions (NR). In non-reducing conditions the Fab is a heterodimer of molecular weight ~50 ku. In reducing conditions the heterodimer dissociates into chimeric light chain (~25 ku) and Fd (~25 ku). Both the gels were stained by silver staining. M is the lane for the protein marker. Similar observations were made in Western Blots (**B**) of the chimeric Fab in reducing (R) and non-reducing (NR) conditions. Electroblotted antibody fragments were detected by Rabbit anti-Human IgG-HRP conjugate (1:1 000 dilution; Dako).

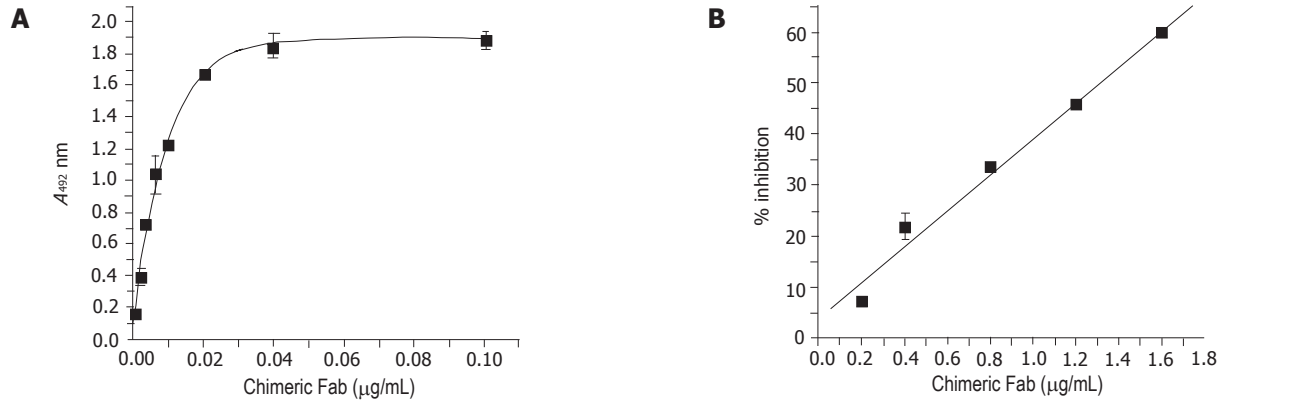

Figure 4 Binding properties of the anti-HBs chimeric Fab. 1 Solid phase ELISA was performed with different dilutions of the soluble chimeric Fab. The result of the competitive ELISA between the chimeric Fab and the mouse monoclonal 5S is shown in Figure (**B**). Different amounts of the chimeric Fab were allowed to compete with 1:80 dilution of the 5S-hybridoma culture supernatant for binding to HBsAg. Bound mouse monoclonal was detected with anti-mouse HRP and the % inhibition of binding was calculated.

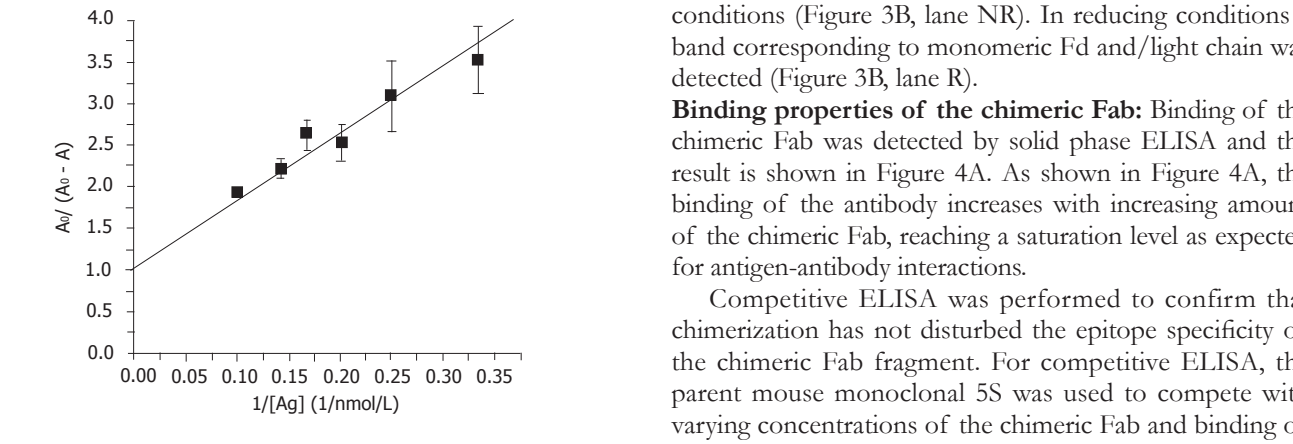

Figure 5 Dissociation constant (K_D) of the chimeric Fab. The K_D was determined using an ELISA based method. Different amounts of HBsAg were incubated with concentrated periplasmic extract of the clone expressing the anti-HBs chimeric Fab for 16 h and unbound antibody was detected by ELISA using rabbit anti-human antibody-HRP (Dako). The data was fitted to the equation $A_0/(A_0–A) = K_D*1/[Ag]+1$, where Ao = absorbance when the antibody was incubated without any antigen, A = absorbance corresponding to free antibody after incubation with the antigen. Dissociation constant of the chimeric Fab was determined from the slope of the straight line (K_D = 8.166±0.14 nmol/L).

conditions (Figure 3B, lane NR). In reducing conditions a band corresponding to monomeric Fd and/light chain was detected (Figure 3B, lane R).

Binding properties of the chimeric Fab: Binding of the chimeric Fab was detected by solid phase ELISA and the result is shown in Figure 4A. As shown in Figure 4A, the binding of the antibody increases with increasing amount of the chimeric Fab, reaching a saturation level as expected for antigen-antibody interactions.

Competitive ELISA was performed to confirm that chimerization has not disturbed the epitope specificity of the chimeric Fab fragment. For competitive ELISA, the parent mouse monoclonal 5S was used to compete with varying concentrations of the chimeric Fab and binding of the mouse antibody was detected using anti-mouse-HRP conjugate. Figure 4B shows that the chimeric Fab inhibits the binding of the mouse antibody, indicating that both of these bind to the same epitope.

Dissociation constant of the chimeric Fab: The dissociation constant of the chimeric Fab in solution phase was determined using an ELISA based technique developed by Friguet *et al.*[36]. As calculated from the slope

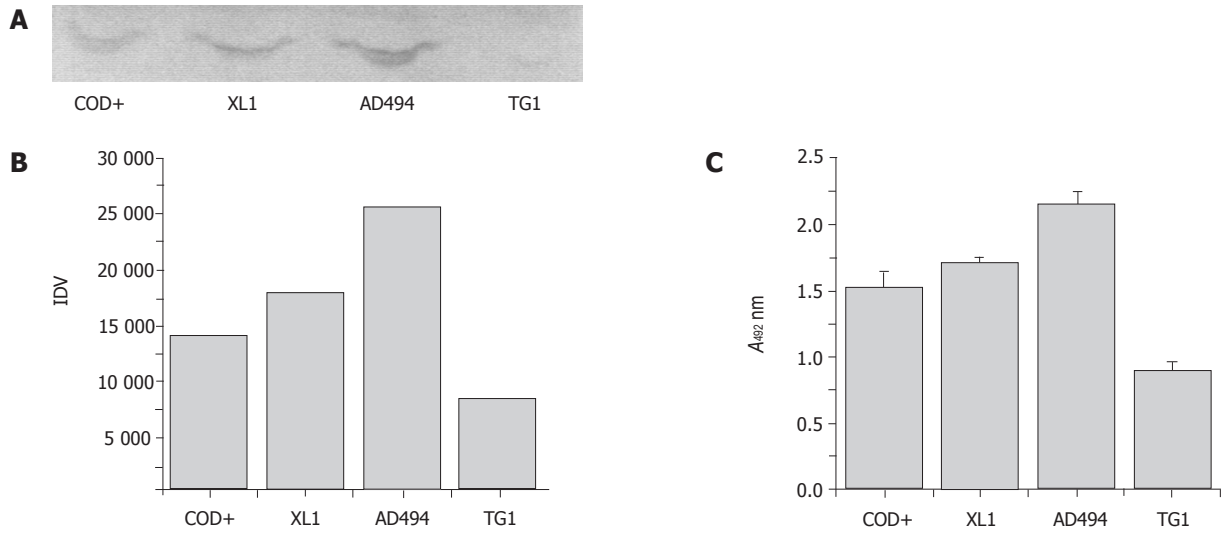

Figure 6 Expression profile of the chimeric Fab in different strains of *E. coli*. The chimeric Fab was expressed in soluble form in different *E. coli* strains in identical conditions. Levels of expression of the chimeric Fab were checked by Western blot in non-reducing conditions (**A**). Intensities of bands were measured densitometrically (**B**). Levels of expression were also checked by ELISA (**C**). Equal amount of the periplasmic extracts were allowed to bind to HBsAg coated on ELISA plate and bound chimeric Fab was detected. (XL1 = XL1-Blue, AD494 = AD494, COD+ = BL21 Codon Plus, TG1 = TG1, IDV = integrated density value).

Table 2 Yields of the anti-HBs chimeric Fab in different *E. coli* strains

E. coli strain	Yield (μg/L)[1]
XL1-Blue	40
AD494	62
BL21 Codon Plus	40
TG1	8

[1]Estimated by Bradford assay, after purification by protein G column

of the line in Figure 5, the K_D of the chimeric anti-HBs Fab is 8.166±0.14 nmol/L, (r^2 = 0.914).

Expression of the chimeric Fab in different *E.coli* strains: Yield of this chimeric Fab in *E. coli* XL1-Blue cells was found to be low (~40 μg/L). The recombinant construct pCOMB3H-Fd-L-Sol was transformed into three other strains of *E. coli*, (AD494, BL21 CodonPlus and TG1) to check the yield of the chimeric Fab in soluble form. The chimeric Fab was expressed in these cells in the same fashion as stated above for XL1-Blue cells. The levels of expression of the chimeric Fab in different *E. coli* strains were checked by Western Blot and compared densitometrically (Figures 6A and B). The levels of expression of the chimeric Fab in different strains of *E. coli* were further confirmed by ELISA using crude periplasmic extracts. The result of the ELISA is shown in Figure 6C. Yields of the purified chimeric Fab expressed in different *E. coli* strains are shown in Table 2. As shown in Figure 6 and Table 2, maximum yield of the chimeric Fab was observed in case of *E. coli* AD494 cells.

DISCUSSION

5S is a mouse monoclonal that binds to HBsAg with high affinity[13]. However, like any other mouse monoclonal, this antibody can give rise to HAMA response and cannot be used clinically. HAMA may neutralize the injected mouse antibody directly by immune complex formation, which could lead to rapid clearance or to hypersensitivity reactions[38,39]. As immunogenic reactions are predominantly directed towards the Fc region of murine antibodies[16], creation of mouse-human chimeric antibodies by swapping murine constant regions with the human ones can address the problem of HAMA response, at least in part[17-19]. But such domain swapping can alter the immunological and pharmacokinetic properties of an antibody, making the task of generation of chimeric antibodies quite tricky[40].

One of the crucial aspects in generation of chimeric antibodies is the selection of human isotypes for domain swapping. Effector functions of antibody constant domains are broadly dependent on the antibody classes and subclasses[41]. Usually human IgG1 is the preferred choice for chimeric antibodies in situations where activation of effector functions is the desired outcome[42]. Rath and Devey[43] had observed that the subclasses of antibodies associated with HBsAg in circulating immune complexes of patients with either acute or chronic HBV infections were predominantly IgG1 and IgG4. A study involving children, who have recovered from acute hepatitis B, has shown that after natural seroconversion, specific antibodies were highly restricted to IgG1[44]. It has also been observed that anti-HBs IgG1 is predominant after vaccination with recombinant vaccines[44,45].

Apart from being important for effector functions, the choice of isotype can also affect the antigen binding of a chimeric molecule. It has been observed that changes in constant domains can affect the functional affinity and specificity of antibodies[46]. Pritsch *et al*.[47] have shown that Fab fragments of antibodies sharing identical variable regions but C_H1 of different isotypes have significant differences in affinities. A molecular dynamic study

involving anti-HEL Fab has shown that, the interactions between C_L and C_H1 domains may have influence not only around the local interface between C_L and C_H1 but also around the interacting regions between HEL and Fv[48].

In the present work, the variable region genes of 5S (IgG1/κ) were fused with the C_H1 region of human IgG1 and human kappa chain constant region, to generate a mouse-human chimeric Fab. The C_H1 region of IgG1 and kappa C_L were amplified using RNA extracted from human PBLs and linked with respective variable regions of 5S hybridoma by overlap PCR. The chimeric fragments were subsequently cloned into the phagemid vector pCOMB3H and phage antibodies were expressed. Functional clones were selected by biopanning over antigen coated plate and selected clones were checked for binding by phage ELISA. It is well known that cloning of antibody genes by PCR may introduce mutations and may amplify truncated antibody genes, generating clones that are non-functional[49,50]. Use of a phagemid expression system allows the initial expression of antibody molecules as phage antibodies, which can be easily selected for antigen binding and then be further processed for soluble expression. As shown in Figure 2, two of the four clones checked by phage ELISA after three rounds of biopanning showed high binding. Clone 1 was further processed for soluble expression of the chimeric Fab. SDS-PAGE and Western blot in both reducing and non-reducing conditions confirmed that the chimeric Fab was expressed in the form of a heterodimer (Figures 3A and B). The chimeric Fab was further characterized by ELISA and competitive ELISA. Competitive ELISA confirmed that the chimeric Fab and the original mouse monoclonal bind to the same epitope (Figure 4B). The dissociation constant of the chimeric Fab as determined by the ELISA based method was found to be high (8.166±0.14 nmol/L). The dissociation constant for the original mouse monoclonal is 0.8899 nmol/L[13]. Given the level of accuracy of the method used for the determination of K_D values, one can consider both of these values to be in the same broad range. An anti-human antibody was used in the ELISA to calculate the K_D of the chimeric Fab; whereas an anti-mouse antibody was used in case of the mouse monoclonal. Such a difference in experimental setup can also be attributed for the difference in K_D values.

Though it seems simple, high yield expression of heterologous proteins in bacterial systems sometimes proves to be problematic. Multiple factors like codon usage[51], DNA/RNA/Protein interaction[52], regulatory factors for transcription and translation and nucleotide usage in the leader peptide[53] affect the yield of heterologous proteins in bacteria. The yield of Fab depends upon the relative levels of expression of the light chain and Fd. The light chain-heavy chain balance depends upon codon usage in the signal peptides and 5' sequence of coding regions[54]. In this present work, the yield of soluble recombinant Fab in XL1-Blue cells was low (~40 μg/L). Such a low yield of Fab fragment expressed in *E. coli* using pCOMB3H vector system has been reported earlier[55]. To optimize the yield of the soluble chimeric Fab, the recombinant construct was transformed in different *E. coli* strains — TG1, AD 494 and BL21 CodonPlus. *E. coli* BL21 CodonPlus-RP (Novagen) cells contain extra copies of the *argU* and *proL* genes. These genes encode tRNAs that recognize the arginine codons AGA, AGG and the proline codon CCC, respectively. *E. coli* BL21 has been used for high-level expression of several antibody fragments[56,57]. *E. coli* AD494 cells are thioredoxin negative (*trxB*$^-$), thereby providing an oxidizing environment in the cytoplasm. Proper folding and assembly of antibody domains require inter- and intra-chain disulphide bond formation. Therefore bacterial periplasm is suitable for the assembly of functional Fab fragments. An oxidizing environment in the cytoplasm may also help to increase the yield of properly folded soluble antibody fragments. We expressed the anti-HBs chimeric Fab in soluble form in different *E. coli* strains under identical conditions. As shown in Figure 6 and Table 2, there was a slight increase in the expression of the chimeric Fab in AD494 cells; whereas the expression in BL21 was equivalent to that in XL1-Blue cells. Though Raffai *et al*.[55] had observed increased yield of soluble Fab fragment in TG1 in comparison to XL1-Blue cells, we could not detect any such increase in yield.

Though chimerization and humanization are essential to reduce immunogenicity of non-human antibodies before they can be used clinically such genetic engineering can alter functional capabilities of the molecule, often leading to loss of binding or reduction in affinity. Our results show that the chimerization of the 5S mouse monoclonal did not disturb its binding to the antigen and the chimeric Fab binds to the same epitope as that of the original mouse monoclonal. Though chimeric and monovalent in nature, the affinity of the recombinant chimeric Fab remained in the same range as the mouse monoclonal 5S. The high affinity binding of this chimeric Fab fragment indicates that it can be further modified to generate a clinically applicable full-length chimeric anti-HBs antibody, with out any significant loss of binding affinity.

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