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(54) Title: A HUMANIZED HIGH AFFINITY RECOMBINANT ANTIBODY AGAINST HEPATITIS B SURFACE ANTIGEN

(57) Abstract: This invention relates to a high affinity recombinant humanized antibody fragment (scFv) specific for hepatitis B surface antigen having unique inter/ intra chain bonding interaction because of 28 altered amino acid residues from the original mouse (5S) antibody and its chimeric Fab form, wherein fine tuning of the vernier zone residue makes it closer to the human sequence without any structural constraints.

Title

A Humanized High Affinity Recombinant Antibody against Hepatitis B Surface Antigen.

Field of Invention

The present invention relates to the generation of high affinity humanized antibody fragment (scFv) against hepatitis B surface antigen for the treatment or prevention of hepatitis B infection.

Background of Invention

HBV (Hepatitis B virus) is a major cause of acute and chronic hepatitis worldwide. Prevention of HBV infection may be achieved with active or passive immunization. Active immunization with recombinant HBV vaccines can prevent HBV infection if given before exposure. Despite the introduction of universal vaccination against hepatitis B in around 100 countries, persistent HBV infection is still a serious problem, with an estimated worldwide death rate of one million people per year. Protective antibodies that appear after natural infection are mostly directed against the major antigenic 'a' determinant of hepatitis B surface antigen (HBsAg). The immunodominant 'a' epitope is present in all serotypes. Antibodies against HBsAg are thus advocated for passive immunotherapy for managing chronic viral infection. Passive immunization with hepatitis B specific antibodies, given shortly after exposure, can decrease the incidence or severity of disease.

Currently, Hepatitis B immune globulin (HBIG), collected from the blood of hyper immune human donors, is used for the post-exposure prophylaxis in cases of accidental needle stick injuries, liver transplant patients and for the prevention of vertical transfer of HBV infection form mother to child. This blood-derived product is not manufactured in India. However, such blood-derived products are costly and can cause cross contamination. Therefore, recombinant antibodies can be good substitutes for human serum antibodies.

Inventors developed a mouse monoclonal antibody (5S) against the 'a' epitope of the hepatitis B surface antigen (HBsAg). However such mouse antibodies often induce a significant human anti mouse antibody (HAMA) response when administered to patients and thus limiting their potential use for human therapy, especially when repeated administration is necessary. HAMA greatly reduces the *in-vivo* efficacy of mouse antibodies. Moreover, the half-life of a mouse antibody in human plasma is shorter compared with that of human antibody. Several recombinant anti-HbsAg antibody fragments have been reported in literature.

However most of them are of mouse origin and are not available for therapeutic purposes (1-15). To reduce the immunogenicity of murine antibodies, chimeric antibodies were constructed, which combine the variable region of a mouse antibody with a human antibody constant region, thus retaining the binding specificity of murine antibody while presenting less foreign amino acid sequence to the human immune

system (16). Inventors also generated chimeric antibody against HBsAg (17-18). Some of the chimeric antibodies have proved less immunogenic in humans, whereas others are almost as immunogenic as murine antibodies. Moreover, in an animal study to evaluate the immunogenicity of chimeric antibodies, it was found that the anti-variable domain response was not attenuated in the chimeric antibodies, suggesting that the murine variable domain can still lead to a significant human anti mouse antibody (HAMA) response (19). Therefore, for therapeutic purposes it may be necessary to fully humanize a murine antibody by altering the variable domain to make them human like. It is well established that humanization of mouse antibody is desirable to reduce its potential product immunogenicity. However humanization is practical only if it does not reduce or destroy the binding affinity of antibody. Humanized antibodies are safer for therapeutic uses and currently several such humanized antibodies are in clinical uses for various diseases. Although some chimeric antibodies are in clinical use, it is worth noting that most of the antibodies in phase I, II and III clinical trials today are humanized antibodies.

All the mouse/humanized and human anti-HbsAg antibodies reported in literature have unique complementarily determining regions (CDRs) sequence and have unique antigen-antibody interactions which are different form the recombinant molecule of this invention.

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Objects of the Invention

The object of the invention is to generate a recombinant humanized antibody fragment (scFv) against hepatitis B surface antigen.

Other object is to develop a humanized recombinant antibody fragment which can bind to hepatitis B surface antigen with high affinity and specificity.

Another object is to produce a recombinant humanized antibody fragment (scFv) which would have less immunogenicity as compared to the mouse monoclonal.

Yet another object to produce a recombinant humanized antibody fragment (scFv) which would be more suitable for *in-vivo* use in humans.

Other objective is to generate anti-HBsAg humanized antibody which can be safer and cheaper alternative

Further object is to develop the recombinant molecule which can also be used with/without any modification/ in combination with other molecules for generation of complete full length antibody, bispecifc antibodies and diabodies or any other modification of a protein molecule with containing the described humanized antibody fragment (scFv).

Yet another object is also to develop immuno-conjugates, like conjugated with enzymes, ligands, receptors, drugs, radio isotopes, toxins or any other large or small molecule for *in-vivo* or *in-vitro* use.

Brief description of the accompanying drawings

Figure1: Shows Schematic representation of generation of single chain variable fragment.

Figure 2: Shows Schematic representation of steps involved in overlap PCR that was used to link gene segments.

Figure3: Demonstrates (a) SDS PAGE analysis of soluble antibody fragment (scFv) expressed in non-suppressor strain of E.coli HB2151. Lane IN AT51 and M represent the periplasmic extract of IPTG induced AT51 clone and protein molecular weight marker respectively.

(b) Western blot analysis of soluble scFv. HB represents the induced periplasmic extract of untransformed HB2151 cells. Antibody fragment was detected using anti E tag antibody followed by anti mouse HRP conjugate.

Figure 4: Demonstrates Antigen binding analysis of soluble recombinant antibody fragment. Similar dilution of periplasmic extract of various clones was used in the experiment. 5S hybridoma soup was used as positive control and periplasmic extract of untransformed HB2151 was used as negative control. AT51 clone was used for further experiments.

Figure 5: Demonstrates ELISA with different dilutions of soluble antibody fragment (scFv). Soluble scFv was used in different dilution to bind to the HBsAg coated on ELISA plate (250ng/well) and bound scFv was detected using anti E tag antibody followed by anti mouse HRP conjugate antibody. Periplasmic extract of untransformed HB2151 cells were used as negative control for the experiment and its binding to the antigen was considered as base line reading for this experiment.

Figure 6: Shows effect of urea on antibody binding, analyzed by ELISA for humanized scFv, mouse scFv and the mouse monoclonal 5S.

Figure 7: Shows effects of DMSO, which destabilizes antigen-antibody interactions, which was checked by ELISA.

Figure 8: Shows effect of NaCl on antigen interaction, which was analyzed by ELISA.

Figure 9. Shows Antigen antibody analysis at different pH by ELISA.

Figure 10: Demonstrates of average dissociation constant (K_D) of 5S mouse monoclonal antibody and humanized scFv. Antibodies were incubated with different amount of the antigen overnight to reach the equilibrium and free antibodies were determined by ELISA with 20 minutes of incubation. Data points were fitted to the equation $A_0/(A_0-A)=K_D[Ag]+1$, where A_0=absorbance when the antibody was incubated without antigen, A=absorbance corresponding to free antibody after incubation with antigen and [Ag]=free antigen concentration which is equal to the antigen taken for experiment considering a pseudo first order reaction. As calculated from the slope of the straight lines, the K_D values for humanized scFv and the mouse monoclonal were 1.206 nM (R^2=0.9872) and 1.114 nM (R^2=0.9909), respectively.

Figure.11: Alignment of 5S VH sequence (a) and 5S VL sequence (b) with the most homologous human consensus sequence (VH subgroup I) and

(VLκ subgroup II) respectively. The mismatched residues are marked in single letter code. CDR regions are marked by box. The sequences of humanized VH and VL are also aligned with their corresponding chains.

Figure.12: Nucleotide sequence of humanized anti-HBs-scFv. Corresponding amino acid sequence is marked in single letter code. The linker peptide is underlined.

Detailed description of the Invention

At the outset

In order to make the humanized antibody fragment (scFv), the inventors have adopted a recombinant approach, where individual V_H and V_L have been linked by flexible linker and cloned in a phagemid vector. For the humanization, non-human like framework residues in the 5S-scFv were selected by aligning them with best homologous human antibody sequence/human consensus sequence. Selected non human like residues were subsequently mutated to human residues by site-directed mutagenesis. In the process of humanization, apart from the residues at low risk positions, several minimal positional residues (high risk positions) were mutated without affecting binding affinity. Interestingly, inventors also did fine tuning of the "vernier zone" residue to get close to the human sequence without and structural constraint. The resulting humanized scFv has generated novel inter/intra-chain bonding interactions as compared to mouse scFv. This humanized scFv shows

high binding affinity and epitope specificity to the HbsAg inspite of the twenty eight altered amino acids. The humanized antibody fragment also has dissociation constant in nano molar range equivalent to that of the original mouse monoclonal.

This recombinant antibody fragment also maintained antigen binding in the presence of various destabilizing agents like 3M NaCl, 30% DMSO, 8M urea and extreme pH. This high affinity humanized scFv provides a platform/basis for the development of therapeutic molecules which can be safely utilized for the treatment of hepatitis B.

At the outset of the description that follows, it is to be understood that the ensuing description only illustrates a particular form of this invention. However, such a particular dorm is only an exemplary embodiment and is not intended to be taken restrictively to imply any limitation on the scope of the present invention.

There are many well known methods to generate humanized antibody. However, every recombinant humanized antibody is unique in nature, if and only if (a) its molecular structure as defined by the amino acid sequence is unique (b) has unique biological function as defined by specificity and affinity for the target antigen. The recombinant molecule of this invention is unique as no other molecule matches the structure and properties of this molecule. This novel molecule can be generated by many other well documented strategies too.

Other recombinant molecule may have the same function as they bind the hepatitis to surface antigen but each molecule with different sequences has unique binding characteristics in terms of epitope specificity i.e. the precise sequence of antigen it binds to and the molecular interactions for doing the same and affinity.

Here, the inventors generated a recombinant humanized antibody fragment (scFv) composed of humanized V_H (variable region of heavy chain) and humanized V_L (variable region of light chain), which binds to hepatitis B surface antigen with high affinity. Only the antibody fragment and not the individual V_L and V_H can bind to hepatitis B surface antigen thus be useful for virus neutralization.

Inventors have used the antibody genes from a mouse monoclonal (5S) for generation of recombinant antibody fragment (scFv) against hepatitis B surface antigen. This antibody binds to the immunodominant 'a' epitope of the hepatitis B surface antigen and found to be protective in a surrogate *in-vitro* assay. This mouse monoclonal was generated using existing protocol for generation of hybridomas. However, this mouse monoclonal can not be used directly in human subjects, as it will induce human anti mouse antibody (HAMA) response. To reduce the immunogenicity of the antibody, inventors have generated a recombinant humanized antibody fragment that retains the high affinity and specificity for HBsAg. Variable region genes (V_H and V_L) of the mouse monoclonal (5S) were amplified by reverse transcription (RT) followed by polymerase chain reaction (PCR). Mouse V_L and V_H were linked with a

flexible linker to generate single chain variable fragment by overlap PCR. Phagemid vector pCANTAB 5E (Amersham Biosceinces) was used to express the soluble scFv in the periplasmic space of *E.coli* (HB2151).

In order to prepare a humanized scFv, we constructed a molecular model of scFv by web based modeling software to analyze the structural significance of each and every residue. We selected a human antibody sequence from database that shows the highest homology of amino acid sequence to the mouse V_H and V_L. The immunogenic (mouse) framework residues were identified that differed from human framework residues in highly homologous human V_H and V_L sequences. The selected residues were subsequently mutated to human residue by site directed mutagenesis. We preserved a few mouse residues based on the information of the possible interaction of these residues with other framework residues observed in a structural model. The selected human antibody sequence contains some unusual residues at certain positions, but the mouse scFv actually has a residue much more typical of human sequences than the selected human antibody. At these positions, we therefore chose to use the parent murine antibody residue rather the selected human antibody residue in the humanized antibody to make the antibody more generically human. These criteria allowed the selection of all amino acids in the humanized scFv as coming from either selected human antibody or form human consensus sequence.

After initial screening using the phage display system, the humanized scFv was expressed in soluble form in the periplasm of *E.coli* (HB2151). The resulting humanized antibody fragment showed high binding to HBsAg and competitive ELISA confirmed that it binds to the same epitope as that of the original mouse monoclonal (5S). The apparent dissociation constant (K_D) of the humanized antibody fragment was found to be very close to that of the original mouse monoclonal (1.206 nM (R^2=0.9872).

This humanized antibody fragment can be further manipulated to generate the complete humanized IgG derivatives by fusing the human heavy and light chain constant domains. Therefore this humanized antibody fragment, which has unique mouse CDRs and human like framework regions can be the starting material for generation of a therapeutically functional full length recombinant antibody which can be utilized for passive therapy in case of HBV infection. Being a humanized antibody, it is expected to be least immunogenic than a chimeric/mouse antibody and a generated by recombinant means it can be safer and cheaper that the currently used human polyclonal antibody.

Cloning and generation of the recombinant antibody fragment (scFv):

The strategy for generation of the recombinant antibody fragment 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 transcription and PCRs are listed in Table 1. Total RNA was extracted from 5S hybridoma cells using standard protocol and cDNA was generated by reverse transcription using a primer against 3' conserved region of antibody variable region genes. PCR amplification of variable region of heavy chain (V_H) and light chain (V_L) was performed with degenerate primers for 35 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 2 min; followed by a final extension at 72°C for 10 min. PCR amplified products were resolved in 1.5% agarose gel and respective bands were eluted out using the standard protocol. For the construction of scFv gene, V_H, V_L and linker fragments were joined by overlap PCR. The strategy for overlap PCR is shown here in figure 2. Initially V_H and linker fragments were taken in equivalent molar ratio and linked by PCR (20 cycles, 50°C annealing) with out using any primer. The PCR product was diluted 10 times and amplified by PCR relation (35 cycles, 50°C annealing) using external primers (46 and 50). Similar protocol was used to join V_L and linker fragment. Both the V_H-linker and linker-V_L fragments were resolved on 1.5% agarose gel and specific bands wer cluted out. These two eluted fragments were linked by another overlap PCR using primers (49 and 45). Assembled scFv was then digested with Not1 and Sfi 1 and ligated into the phagemid vecot pCANTAB 5E, which includes the 'E tag' for detection and purification purposes. XL-1-Blue cells were transformed with the resulting phagemid pCANTAB-5S-scFv using a standard chemical ($CaCl_2$) transformation protocol. Transformed cells were grown on Ampicillin-Agar plates. Colonies wer picked up after

overnight incubation and screened for presence of the insert by PCR screening and restriction digestion.

Selection and expression of antigen binding antibody fragment:

It is well known that truncation and mutations can be generated due to PCR and the cloning process. Therefore, cloned scFv library was screened by bio-panning over antigen coated ELISA plate. After three rounds of enrichment, selected colones were used to prepare phage antibody and antigen-binding colones were detected by phage-ELISA Clone AT51 which showed the maximum absorbance has been used for soluble expression by inducing $HB2151^{Nal}$ culture with 1mM IPTG for 6 h at 27°C. After induction, a band with molecular weight of ~29 kDa, corresponding to scFv was detected in the periplasmic extract of AT51 infected $HB2151^{Nal}$ cells (Fig. 3a), this was further confirmed by western blot using HRP/anti-E tag mouse antibody (Fig. 3b). Binding of the recombinant antibody fragment was detected by solid phase ELISA and the result is shown in Figure 4.

Molecular modeling of recombinant antibody fragment:

In order to study the importance of the framework residues that could influence CDR conformation, and thus Ag-binding affinity and/or activity. Molecular model of the scFv was constructed using WAM, web based antibody modeler. The model of humanized scFv was further constructed by WAM. Single residue changes were made manually in the

resulting model and then subjected to energy minimization using the software provided with the Swiss PDB Viewer program. The compatibility of all the substitutions in framework regions with remaining structure was analyzed. We superimposed the models of mouse and humanized scFv and systematically compared each and every residue. The quality of molecular models of mouse 5S-scFv and humanized scFv was determined by examining the distribution of amino acids residues in the Ramachandran plot.

Humanization of the antibody fragment (5S-scFv):

The amino acid sequences of V_H of 5S-scFv were independently aligned against the entire repertoire of human antibody sequences contained in the Gene Bank database using BLAST search. The inventors considered human consensus sequences and best homologous human antibody sequences (depending upon the sequence similarity) for humanization of 5S-scFv, as it was showing highest homology with the human consensus sequences tabulated in the database. The human antibodies chosen also had similarity to 5S-scFv in the sequence of the CDRs and had the same loop length (except CRD H3), which further indicates that they belongs to a similar structural group and perhaps have a similar canonical structure of CDR loops.

In order to humanize the recombinant mouse antibody fragment, non-human like framewok residues in the 5S-scFv were selected by aligning

them with best homologous human antibody sequence/human consensus sequence. Among the selected residues, only those were mutated which were not showin any structgural discrepancy with in the context of molecular model of 5S-scFv. Selected non-human like residues were subsequently mutated to human residues using the pCABTAB 5E vector containing mouse 5S-scFv DNA with the QuickChange Multi Site-Directed Mutagenesis kit (Stratagene) according to the manufacture's instructions. All the primers used in site-directed mutagenesis are listed in Table No.2A and 2B. Individual colonies of mutant clones wer sequenced to confirm the presence of the correct mutations.

Binding properties of the humanized antibody fragment:

Antigen binding assay for the humanized antibody fragment was done by solid phage ELISA (Figure. 5). As shown in the figure 5, the binding of humanized scFv increase with the increasing amount of the humanized scFv and reaching a saturation level as expected in the antigen-antibody interactions. Further characterization of binding strength and conformational stability of both the molecules was evaluated by ELISA in the presence of different concentration of various destabilizing agents like urea, DMSO, NaCl and at different pH. As shown in Figure 6 and 7 both mousse scFv and the humanized scFv bind to the antigen in higher concentration of urea and DMSO like mouse monoclonal (5S), indicating strong and very stable interaction between the antigen and antibodies. Similar results were obtained in presence of different concentration of NaCl and at extrement of pH as shown in Figure 8 and 9.

Dissociation constant of the mouse 5S-scFv and humanized scFv:

Dissociation constant K_D, for mouse 5S-scFv and the humanized scFv were determined by the standard ELISA based method. To meet the basic assumptions of the method, antigen was taken in excess to the antibody and all experiments were performed within the range of dilutions of the antibody, where absorbance in the ELISA changes linearly with dilution. As the interaction between coated antigen with the free/bound antibody can shift the equilibrium, we had incubated the overnight equilibrated antigen-antibody mixture, in antigen coated plate only for 20 min. This time is sufficient enough to measure the free antibody without causing considerable shift in the equilibrium (data not shown). The K_D values can be calculated from the slope of the straight lines in Figure 10. The experiments show that the humanization process did not undermine the binding affinity and conformational stability of humanized scFv. The humanized scFv has shown similar binding and kinetic properties as of the present murine monoclonal.

Inventive Steps:

1. Amplification of the variable region genes (V_H and V_L) of the anti-HBs mouse monoclonal 5S by reverse transcription (RT) followed by polymerase chain in reaction (PCR). RNA isolated form 5S hybridoma was used as the source of V_H and V_L genes.
2. Generation of recombinant antibody fragment (scFv) by joining V_L and V_H with a flexible linker by overlap PCR.

3. Recombinant antibody fragment (scFv) was cloned into pCANTAB 5E phagemid vector (Amersham Biosciences) for expression of phage antibody (E.coli; XL1-blue, suppressor strain) as well as soluble scFv (E.coli; HB2151, nonsuppressor strain).
4. Selection of best homologous human antibody sequence from the database in order to humanize the recombinant mouse scFv.
5. Structural significance of each and every residue of mouse scFv was analyzed with the help of computer aided molecular modeling.
6. Selected mouse frame work residues were subsequently mutated to human residue by site directed mutagenesis.
7. Binding analysis and affinity measurement of recombinant humanized antibody fragment (scFv) was done by various ELISA based methods.

Table.1: List of primes used in reverse transcription and PCR

Light chain 3' (45)	5' -TCG ACT TGC GCC CGC CCG TTT KAK YTC CAR CTT KGT SCC-3'
Heavy chain 3'primer (47)	5'-TGA RGA GAC RGT GAC TGA RGT-3'
Light chain 5' primer (44)	5' ATT GTG ATG ACC CAG ACT-3'
Heavy chain 5' primer (46)	5' GCA ACT GCG GCC CAG CCG GCC ATG GCC GAG GTG CAG CTK CAG CAG-3'
Linker template (48)	5' GGT GGT GGT GGG AGC GGT GGT GGC ACT GGC GGC GGC GGA TCT-3'
Linker template 5' (49)	5' TCA GTC ACY GTC TCY TCA GGT GGT GGT GGG AGC-3'
Linker template 3' (50)	5' GT CTG GGT CAT CAC AAT AGA TCC GCC GCC GCC-3'

Present invention deals with a high affinity humanized antibody fragment specific for hepatitis B surface antigen. This recombinant molecule has unique inter/intra chain bonding interaction as it has several altered amino acid residues form the original mouse (5S) antibody and its chimeric Fab from (Patent Application #2704/DEL/2006). Interestingly, inventors also did fine tuning of the "vernier zone" residue to get close to the human sequence without any structural constraint. Vernier residues are known fro making direct contact with the antigen and/or for making V_H /V_L domain interface. This recombinant humanized scFv is unique molecule in terms of antigen contact and structural base from any other known anti HbsAg antibody available in the literature.

Currently, Hepatitis B immune globulin (HBIG), collected form the blood of hyper immune human donors, is used for the post-exposure prophylaxis in cases of accidental needle stick injuries, liver transplant patients and for the prevention of vertical transfer of HBV infection form mother to child. This blood-derived product is not manufactured in India. However, such blood-derived products are costly and can cause cross-contamination. Therefore a recombinant antibody to HbsAg can be suitable alternatives to such a practice. Inventors have developed a mouse monoclonal antibody (5S) against the 'a' epitope of the hepatitis B surface antigen (HBsAg). However such mouse antibodies often induce a significant human anti mouse antibody (HAMA) response when administered to patients and thus limiting their potential use for human therapy, especially when repeated administration is necessary.

For therapeutic purposes it may be necessary to fully humanize a murine antibody by altering the variable domain to make them human like. It is well established that humanization of mouse antibody is desirable to reduce its potential product immunogenicity. However humanization is practical only if it does not reduce or destroy the binding affinity fo antibody. Humanized antibodies are safer for therapeutic uses and currently several such humanized antibodies are in clinical uses for various diseases. Although some chimeric antibodies are in clinical use. It is worth noting that most of the antibodies in phase 1, II clinical trials today are humanized antibodies.

The anti-HBsAg humanized antibody fragment invented can be further manipulated and can be utilized for passive therapy in case of HBV infection. The humanized antibody is safer and cheaper alternative and more suitable for therapeutic use.

Table: 2A

List of primers used in site-directed mutagenesis of VH gene fragment

Position	Mutation	Primer Sequence
H5	(Q-V)	5'-G GTG CAG CTG CAG **GTG** CCC GGG GCT GAG-3'
H7	(P-S)	5'-G CAG CTG CAG CAG **AGC** GGG GCT GAG C-3'
H11	(L-V)	5'-GGG GCT GAG **GTG** GCG ACG CCT GG-3'
H13	(T-K)	5'-GCT GAG CTG GCG **AAG** CCT GGG GCC TC-3'
H18	(L-V)	5'-CT GGG GCC TCA **GTG** AAG ATG TCC TGC AAG-3'
H20	(M-V)	5'-G GCC TCA GTG AAG **GTG** TCC TGC AAG G-3'
H38	(K-R)	5'-CAC TGG GTA **CGG** CAG ACA CCT GG-3'
H40	(T-A)	5'-G GTA CGG CAG **GCA** CCT GGA CGG GG-3'
H43	(R-Q)	5'-G GCA CCT GGA **CAG** GGC CTG GAA TG-3'
H75	(S-T)	5'-CT GCA GAC AAA TCC **ACC** AGC ACA GCC TAT TTG C-3'
H81	(H-E)	5'-C TCC AAC ACA GCC TAT TTG **GAA** CTC AAC AGC CTG ACA TC-3'
H82a	(N-S)	5'-CC TAT TTG GAA CTC **AGC** AGC CTG ACA TCT G-3'
H83	(T-R)	5'-C AGC AGC CTG **AGA** TCT GAG GAC TC-3'
H87	(S-T)	5'- G ACA TCT GAG GAC **ACC** GCG GTC TAT TAC TG-3'
H108	(S-L)	5' -GGT CAA GGA ACC **CTG** GTC ACT GTC TCT TC- 3'

Table: 2B

List of primers used in site-directed mutagenesis of VL gene fragment

Position	Mutation	Primer Sequence
L7	(T-S)	5'-GTG ATG ACC CAG **AGT**_CCA CTC TCC C-3'
L14	(S-T)	5'-CC CTG CCT GTC **ACC**_CTT GGA GAT CAA GC-3'
L15	(L-P)	5'-C CTG CCT GTC ACC **CCG**_GGA GAA CAA GCC T-3'
L17	(D-E)	5'-CT GTC ACC CTT GGA **GAA**_CAA GCC TCC ATC TC-3'
L18	(Q-P)	5'-C ACC CCG GGA GAA **CCG**_GCC TCC ATC TCT T-3'
L36	(H-Y)	5'-CC TAT TTG GAA TGG **TAC**_CTG CAG AAA CCA G-3'
L45	(K-Q)	5' -GGC CAG TCT CCA **CAG** CTC CTG ATC TAC-3'
L70	(E-D)	5' -GT GGA TCA GGG ACA **GAT** TTC ACA CTC AAG-3'
L83	(L-V)	5'-GGA GGC ACC AAG **GTG** GAA CTC AAA CGG GC-3'
L87	(F-Y)	5' -GAT GTG GGA GTT TAT **TAC** TGC TTT CAA CGT TC -3'
L100	(G-Q)	5'-G TGG ACG TTC GGT **CAA** GGC ACC AAG CTG-3'
L104	(L-V)	5'-GGA GGC ACC AAG **GTG** GAA CTC AAA CGG GC-3'
L106	(L-I)	5'-GC ACC AAG GTG GAA **ATC** AAA CGG GCG G-3'

We claim:

1. A high affinity recombinant humanized antibody fragment (scFv) specific for hepatitis B surface antigen having unique inter/intra chain bonding interaction because of 28 altered amino acid residues from the original mouse (5S) antibody and its chimeric Fab form, wherein fine tuning of the vernier zone residue makes it closer to the human sequence without any structural constraints.

2. A high affinity recombinant humanized antibody fragment as claimed in claim 1, wherein the critical residues for antigen binding and protein folding (including the vernier residues) are either identified and retained or modified to amino acids that will retain the key antigen binding characteristics of the molecule and yet convert the protein to one very closely resembling the homologue of human origin.

3. The high affinity recombinant humanized antibody fragment as claimed in claim 1, wherein the said molecule is unique in terms of amino acid sequence, antigen contact and structural base from any other known anti HbsAg antibody.

4. The high affinity recombinant humanized antibody fragment as claimed in claim 1, wherein the immunogenicity to humans is reduced as compared to a mouse or chimeric antibody and hence would be better for all *in vivo* use.

5. The high affinity recombinant humanized antibody fragment as claimed in claim 1, wherein it is safer and cheaper than currently used human polyclonal antibody , as it is generated by recombinant means.

6. The high affinity recombinant humanized antibody fragment as claimed in claim 1 wherein it, as whole or a significant part thereof, can be used for generating all kinds of possible molecules including full length antibody, Fab fragments, chimeric proteins and conjugates of the same with other compounds and structures and with therapeutic entities, toxins or imaging modalities.

7. The high affinity recombinant humanized antibody fragment as claimed in claim 1, wherein it can be used either on its own or as a starting material for another molecule, with or without appropriate modifications or conjugations for developing a diagnostic reagent for the detection of hepatitis B surface antigen.

AMENDED CLAIMS
received by the International Bureau on 22 June 2009 (22.06.2009)

Claims:

1. A humanized antibody fragment which specifically binds to Hepatitis B surface antigen (HBsAg), and comprising:

   (a) at least part of a human immunoglobulin framework region and at least part of a non-human complementarity determining regions (CDRs), said antibody capable of binding to Hepatitis B surface antigen (HBsAg), wherein the CDRs comprises amino acid sequences SEQ ID NO: 1,2,3,4,5 and SEQ ID NO:6

   (b) framework regions in which the non-human amino acids were substituted by human amino acid residues at sites selected from the most abundant human immunoglobulins, comprising the amino acid sequences of SEQ. ID NO:7,8,9,10,11,12,13 and 14

   (c) non-human Complementarily Determining Region (CDR) amino acid residues which binds to Hepatitis B surface antigen (HBsAg) incorporated into a human antibody variable domain, and further comprising a Framework Region (FR) amino acid substitution at a site selected from the most abundant human immunoglobulins consisting of: 7L, 14L, 15L, 17L, 18L, 36L, 45L, 70L, 83L, 87L, 100L, 104L, 106L, 5H, 7H, 11H, 13H, 18H, 20H, 38H, 40H, 43H, 75H, 81H, 82aH, 83H, 87H, and 108H utilizing the numbering system set forth in Kabat numbering convention (Table 3 and 4)

   (d) humanized Framework Region (FR) which were modified by total 28 amino acid substitution at a site selected from the most abundant human immunoglobulins consisting of: 7L, 14L, 15L, 17L, 18L, 36L, 45L, 70L, 83L, 87L, 100L, 104L, 106L, 5H, 7H, 11H, 13H, 18H, 20H, 38H, 40H, 43H, 75H, 81H, 82aH, 83H, 87H, and 108H utilizing the numbering system set forth in Kabat numbering convention, wherein the framework residue is selected from the group consisting of :(i) a residue that non-covalently binds antigen directly;(ii) a residue adjacent to a CDR;(iii) a CDR-interacting residue; and (iv) a residue present in the VL–VH interface

2. A humanized antibody fragment according to claim 1, wherein the 28 framework residue are substituted from the most abundant human immunoglobulins, the residues which are critical for binding and which have a homologous human counterpart that has been identified by molecular model based on the solved structure of immunoglobulins and experimentally verified are as described in (Table 3 and 4).

3. A humanized antibody fragment according to claim 1, wherein the 28 framework residues are substituted as described in Table 3 and 4, wherein the binding properties which reflect the nature of high affinity antigen binding are as follows:

   (a) humanized antibody fragment binds to the recombinant HBsAg and HBsAg derived from human serum of recovered patients with an apparent dissociation constant (K_D) of about 1.20 nM or better affinity determined by ELISA based method

   (b) humanized antibody fragment binds to the recombinant HBsAg and HBsAg derived from human serum of recovered patients in the presence of 4M Urea, 2M NaCl, 20% DMSO and at pH range 6 to 9

4. A humanized antibody fragment according to claim 1, wherein framework region of VL (variable domain of light chain) has 100% homology to the chosen human consensus framework sequence (V kappa II), whereas, framework region of VH (variable domain of heavy chain) has 91% homology to the human subgroup framework sequence (V_HI), wherein the closeness of humanized antibody fragment, to the human sequence reduces the proportion of non-self immunogenic sequences as determined by homology comparisons, the reduction in immunogenicity is consequences of increase in percentage homology (in the framework region) with the human sequences which has increased from 84% to 91% (in case of VH) and 83% to 100% (in case of VL)

5. A humanized antibody fragment according to claim 1, wherein, it as a whole or its Complementarity Determining Region (CDRs) can be used for generating full length antibodies with different effector functions like IgG1 and its subclasses and other immunoglobulin types.

6. A humanized antibody fragment according to claim 1, wherein it can be used as diagnostic reagent for the detection of Hepatitis B surface antigen (HBsAg), wherein the utility of this humanized antibody fragment for HBsAg binding is based on its effective performance in the ELISA.

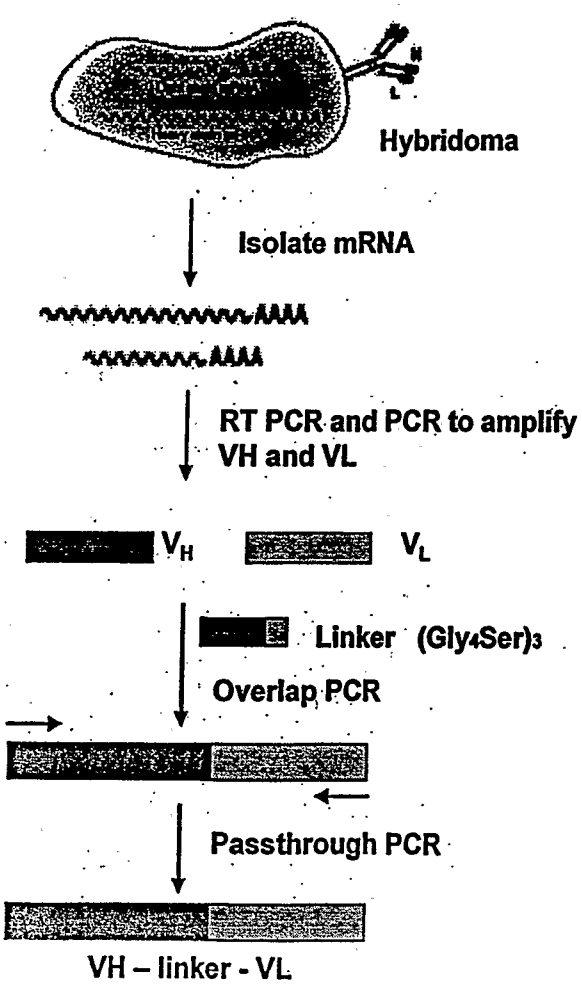

Figure.1

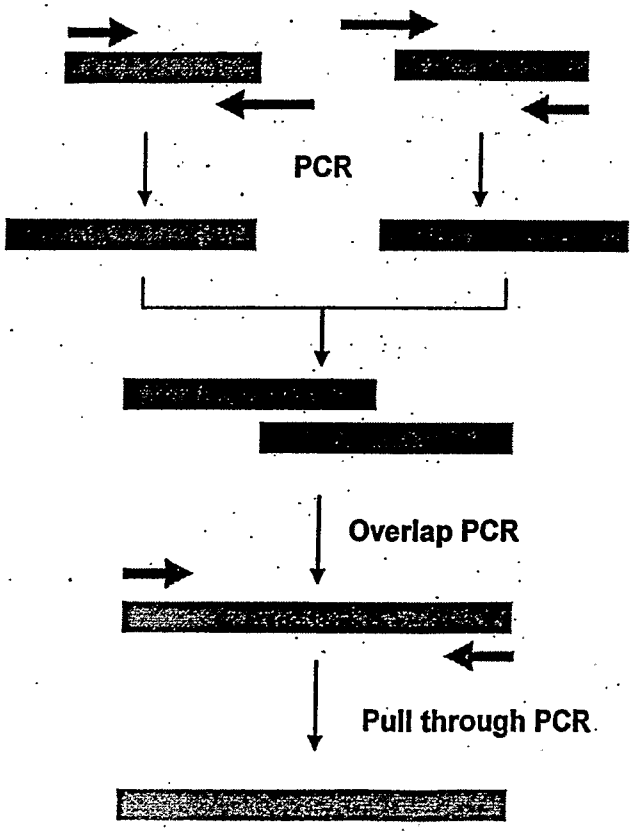

Figure.2

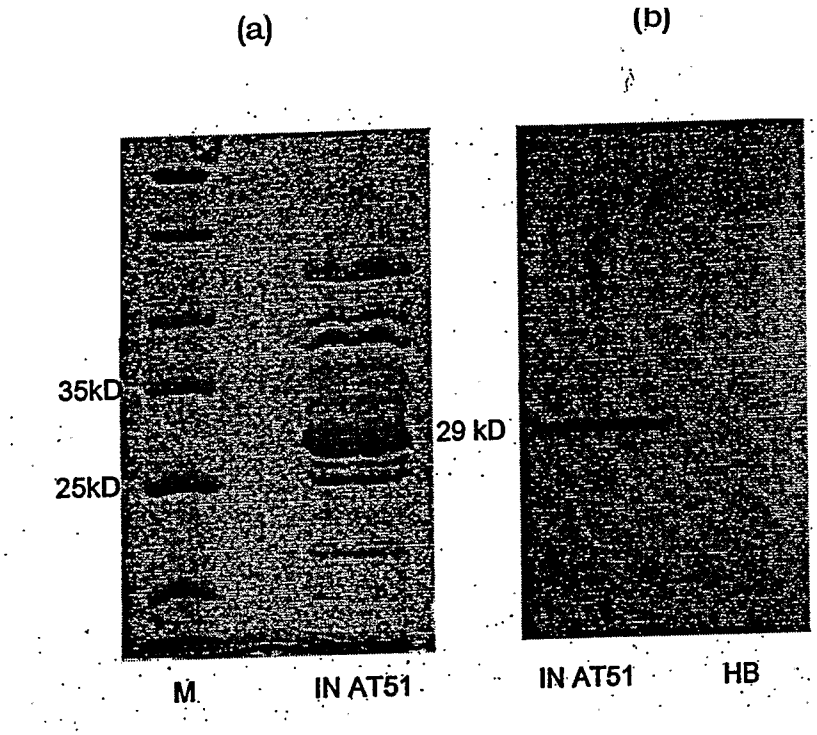

Figure 3

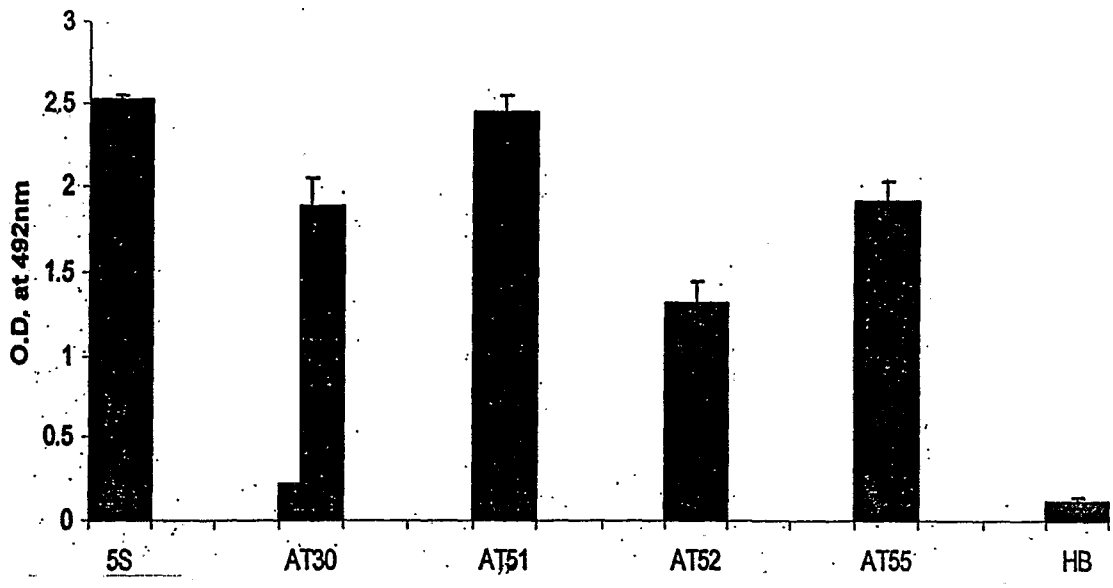

Figure 4

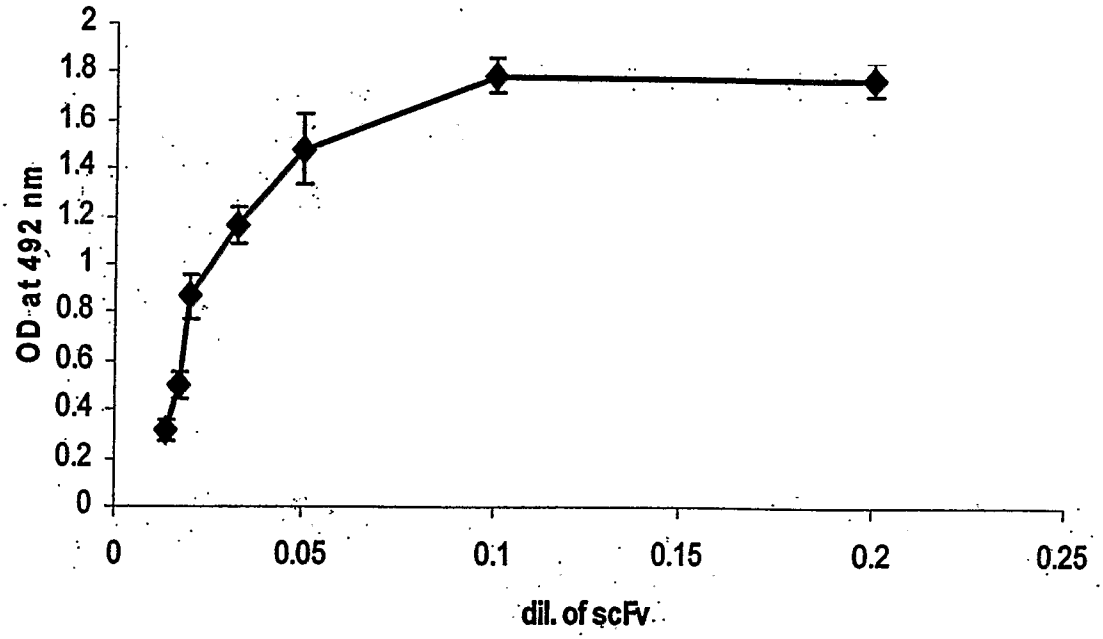
OD at 492 nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.05
0.1
0.15
0.2
0.25
dil. of scFv

Figure 5

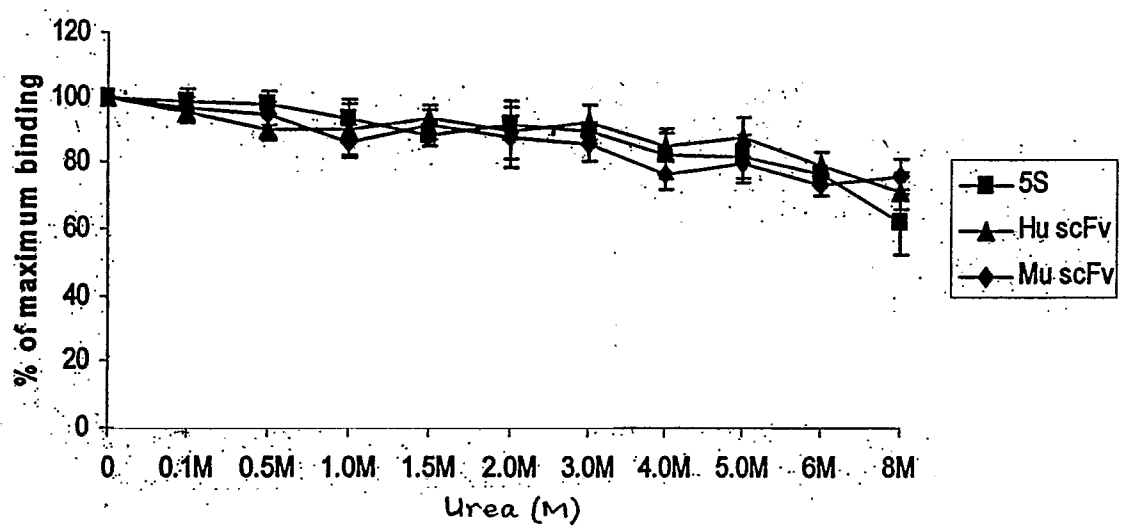
120
100
80
60
40
20
0
% of maximum binding
0
0.1M
0.5M
1.0M
1.5M
2.0M
3.0M
4.0M
5.0M
6M
8M
Urea (M)
5S
Hu scFv
Mu scFv

Figure 6

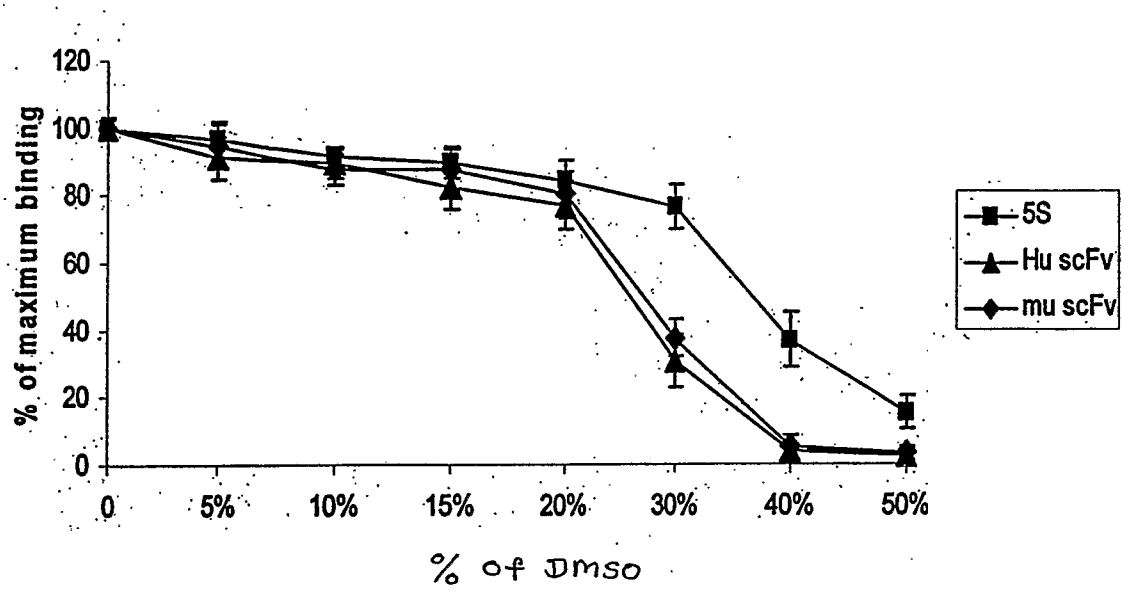
120
100
80
60
40
20
0
% of maximum binding
0
5%
10%
15%
20%
30%
40%
50%
% of Dmso
5S
Hu scFv
mu scFv

Figure 7

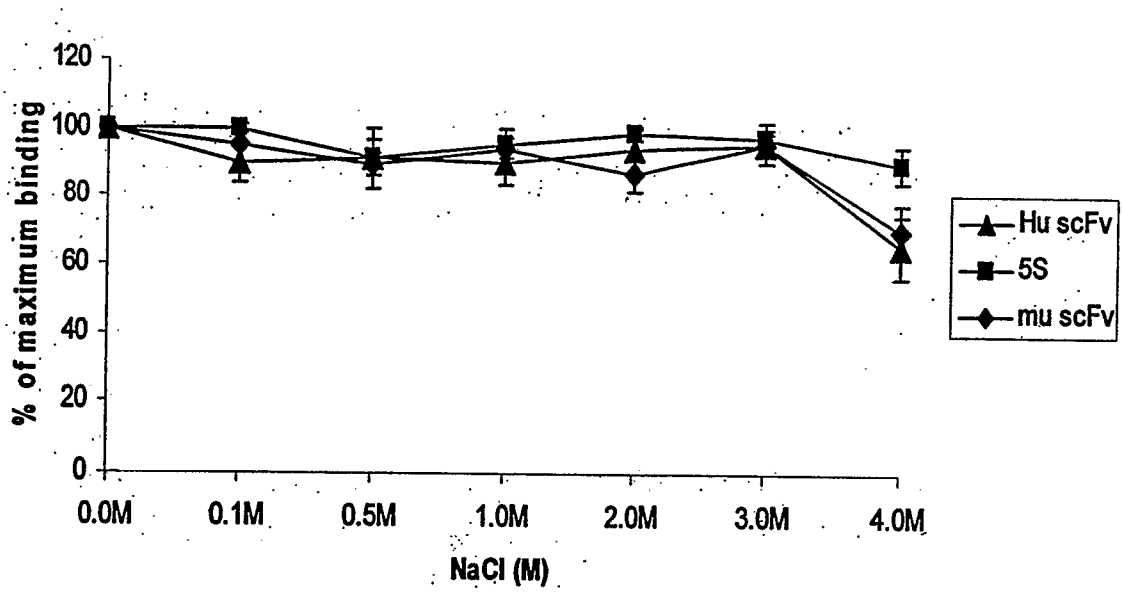
120
100
80
60
40
20
0
% of maximum binding
0.0M
0.1M
0.5M
1.0M
2.0M
3.0M
4.0M
NaCl (M)
Hu scFv
5S
mu scFv

Figure 8

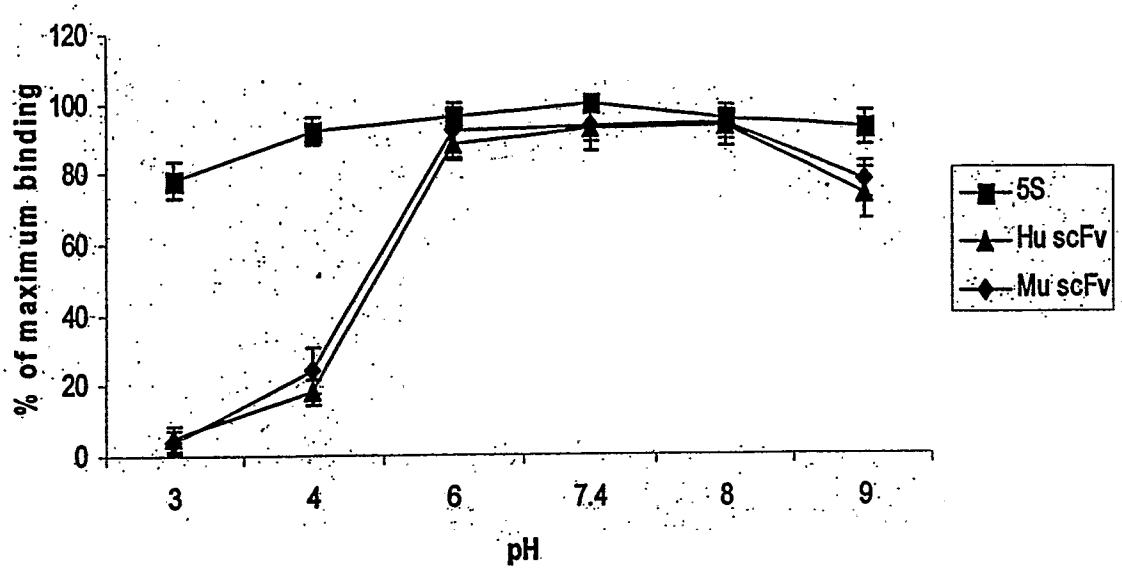
120
100
80
60
40
20
0
% of maximum binding
3
4
6
7.4
8
9
pH
5S
Hu scFv
Mu scFv

Figure 9

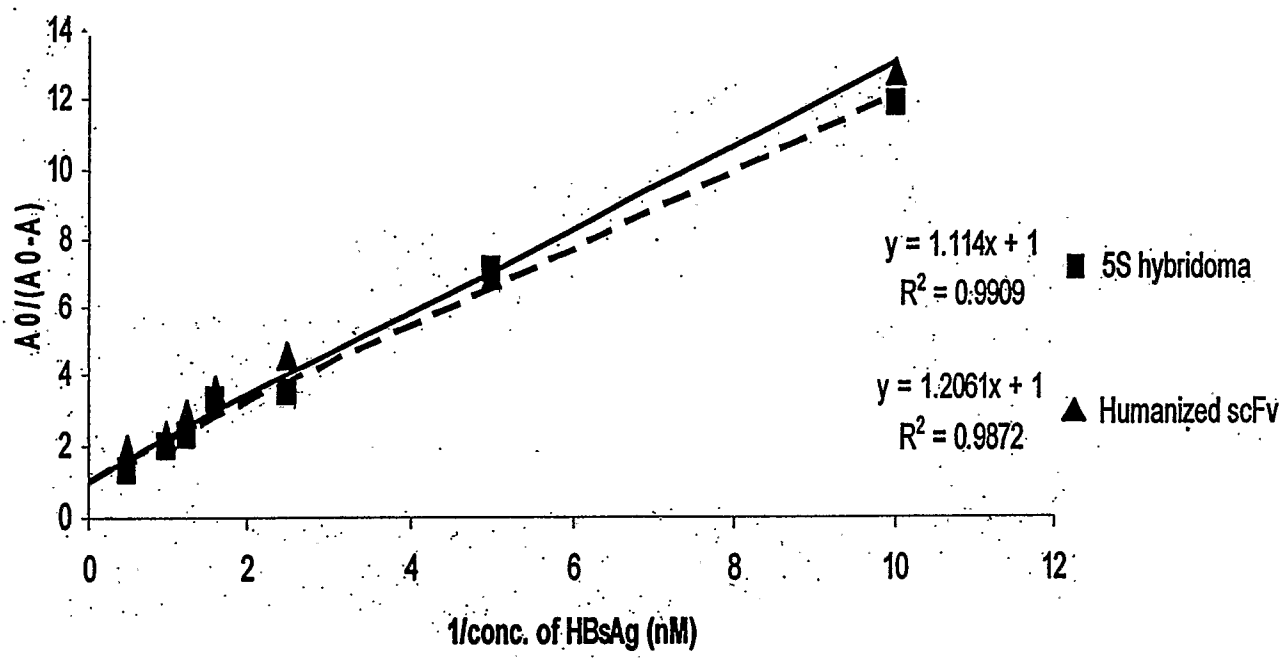
A0/(A0-A)
1/conc. of HBsAg (nM)
y = 1.114x + 1
R2 = 0.9909
5S hybridoma
y = 1.2061x + 1
R2 = 0.9872
Humanized scFv

Figure 10

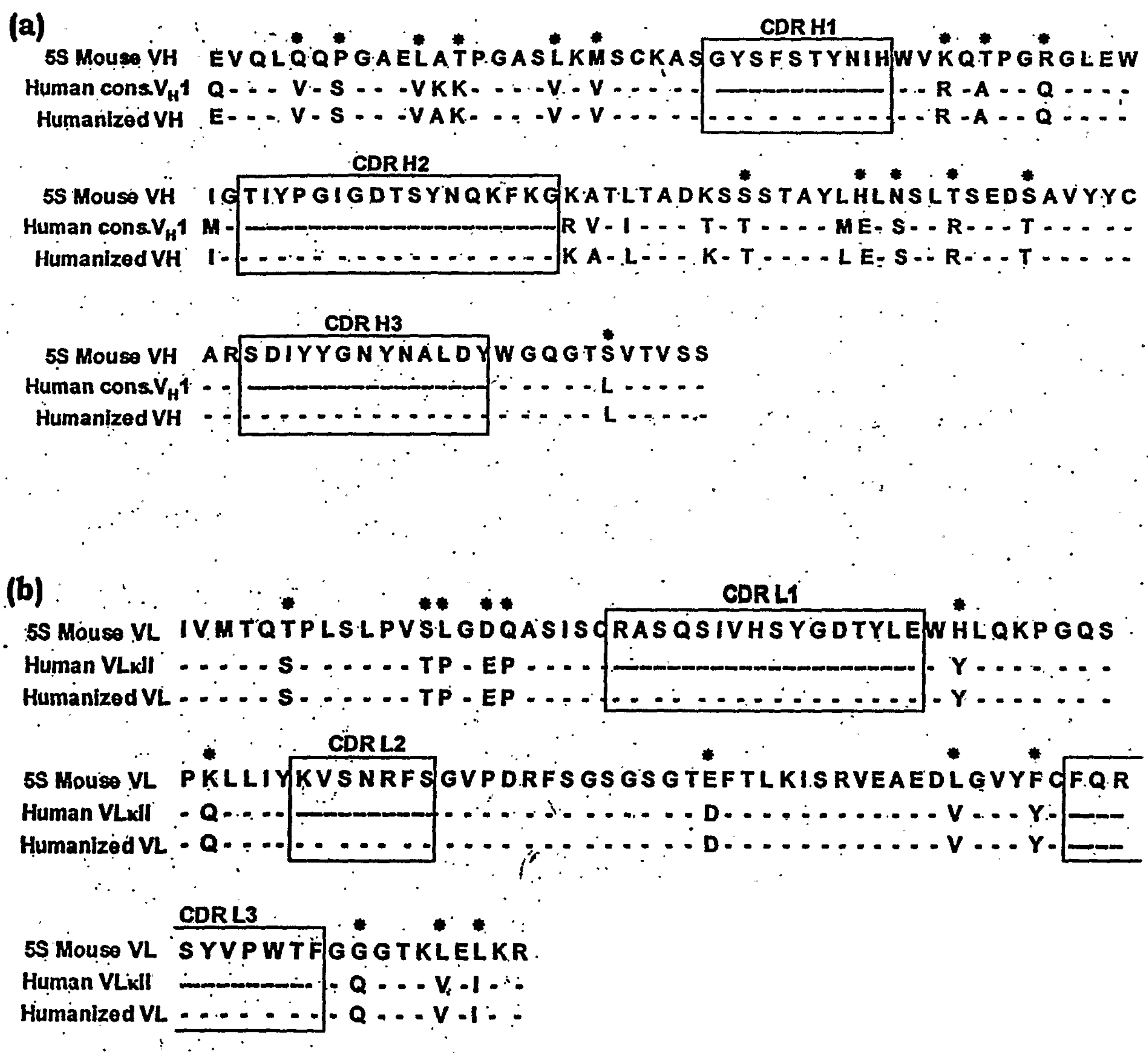
(a)
CDR H1
5S Mouse VH EVQLQQPGAELATPGASLKMSCKASGYSFSTYNIHWVKQTPGRGLEW
Human cons.VH1 Q---V-S---VKK----V-V-----R-A--Q----
Humanized VH E---V-S---VAK----V-V-----R-A--Q----
CDR H2
5S Mouse VH IGTIYPGIGDTSYNQKFKGKATLTADKSSSTAYLHLNSLTSEDSAVYYC
Human cons.VH1 M-RV-I---T-T----ME-S--R---T-----
Humanized VH I-KA-L---K-T----LE-S--R---T-----
CDR H3
5S Mouse VH ARSDIYYGNYNALDYWGQGTSVTVSS
Human cons.VH1 -------L-----
Humanized VH -------L-----
(b)
CDR L1
5S Mouse VL IVMTQTPLSLPVSLGDQASISCRASQSIVHSYGDTYLEWHLQKPGQS
Human VLκII -----S------TP-EP-----Y-------
Humanized VL -----S------TP-EP-----Y-------
CDR L2
5S Mouse VL PKLLIYKVSNRFSGVPDRFSGSGSGTEFTLKISRVEAEDLGVYFCFQR
Human VLκII -Q-----------D-----------V---Y-
Humanized VL -Q-----------D-----------V---Y-
CDR L3
5S Mouse VL SYVPWTFGGGTKLELKR
Human VLκII -Q---V-I--
Humanized VL -Q---V-I--

Figure 11

```
atggcggaagtgcagctggttcagagcggtgcggaagtggcgaaaccgggtgcgagcgtg
 M  A  E  V  Q  L  V  Q  S  G  A  E  V  A  K  P  G  A  S  V
aaagtgagctgcaaagcgagcggctatagctttagcacctataacattcattgggtgcgt
 K  V  S  C  K  A  S  G  Y  S  F  S  T  Y  N  I  H  W  V  R
caggcgccgggtcagggcctggaatggattggcaccatttatccgggcattggcgatacc
 Q  A  P  G  Q  G  L  E  W  I  G  T  I  Y  P  G  I  G  D  T
agctataaccagaaattcaaaggcaaagcgaccctgaccgcggataaaagcaccagcacc
 S  Y  N  Q  K  F  K  G  K  A  T  L  T  A  D  K  S  T  S  T
gcgtatctggaactgagcagcctgcgtagcgaagataccgcggtgtattattgcgcgcgt
 A  Y  L  E  L  S  S  L  R  S  E  D  T  A  V  Y  Y  C  A  R
agcgatatttattacggcaactacaacgcgctggattattggggccagggcaccctggtt
 S  D  I  Y  Y  G  N  Y  N  A  L  D  Y  W  G  Q  G  T  L  V
accgtgagcagcagcggcggtggtagcggtggtggtggcaccggtggtggcggcagcatt
 T  V  S  S  S  G  G  G  G  S  G  G  G  G  T  G  G  G  G  S  I
gtgatgacccagtctccgctgagtctgccggttacgccgggtgagccggccagcattagc
 V  M  T  Q  S  P  L  S  L  P  V  T  P  G  E  P  A  S  I  S
tgccgtgcgagccagagcattgtgcatagctatggcgatacctatctggaatggtatctg
 C  R  A  S  Q  S  I  V  H  S  Y  G  D  T  Y  L  E  W  Y  L
cagaaaccgggtcagtctccgcagctgctgatttataaagtgagcaaccgttttagcggc
 Q  K  P  G  Q  S  P  Q  L  L  I  Y  K  V  S  N  R  F  S  G
gtgccggatcgctttagcggcagcggtagtggcaccgattttaccctgaaaattagccgt
 V  P  D  R  F  S  G  S  G  S  G  T  D  F  T  L  K  I  S  R
gtggaagcggaagatgtgggcgtgtattattgttttcagcgtagctatgtgccgtggacc
 V  E  A  E  D  V  G  V  Y  Y  C  F  Q  R  S  Y  V  P  W  T
tttggccagggcaccaaagtggaaattaaacgt
 F  G  Q  G  T  K  V  E  I  K  R
```

Figure 12

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IN 2008/000796

A. CLASSIFICATION OF SUBJECT MATTER
IPC[8]: ***C07K 16/08*** (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[8]: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Wpi, Epodoc, Internet, Pubmed, Registry, Medline, Genbank, Uniprot, EBI-Patent, NCBI-Patent

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Bose B, Chugh DA, Kala M, Acharya SK, Khanna N, Sinha S. "Characterization and molecular modeling of a highly stable anti-Hepatitis B surface antigen scFv." Mol Immunol. 2003 Dec;40(9):617-31. **Whole document**	1
Y	GENBANK Accession: CAD70712, Version CAD70712.1 GI:28950508, "anti-HBsAg ScFv antibody [synthetic construct]" 21 November 2003 (21.11.2003) . [DB Source: embl accession AJ549501.1] **Whole document**	1

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 March 2009 (25.03.2009)	21 April 2009 (21.04.2009)
Name and mailing address of the ISA/ AT **Austrian Patent Office** Dresdner Straße 87, A-1200 Vienna Facsimile No. +43 / 1 / 534 24 / 535	Authorized officer GÖRNER W. Telephone No. +43 / 1 / 534 24 / 558

Continuation of first sheet

Continuation No. I:

Nucleotide and/or amino acid sequence(s)

(Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material: a sequence listing

b. format of material: a sequence listing

Continuation No. II:

Observations where certain claims were found unsearchable

(Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

Claims Nos.: 2-7 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

Claim 1 is not clear and concise since it does not provide any meaningful technical features describing and defining the recombinant humanized antibody fragment (scFv) such as its sequence, only the hint that 28 amino acid thereof are altered. The search as well as the establishment of novelty, inventive step and industrial applicability was nevertheless carried out based on the peptide according to the amino acid sequence from Figure 12 of the present application.

Claim 2 is unclear since it refers in general to critical residues in antigen binding and folding which "will retain key antigen binding characteristics of the molecule and yet convert the protein to one very closely resembling the homologue of human origin", none of which residues is described clearly and concisely. Furthermore, the category of claim 2 is unclear since it is a product claim which contains process features.

Claim 3 is unclear since it is directed to a recombinant humanized antibody fragment which generally "is unique in terms of amino acid sequence, antigen contact and structural base from any known anti-HbsAg antibody" and thus does not contain any defined clear and concise technical features.

Claim4 is unclear since the general reference to any mouse or chimeric antibody for

comparison in terms of immunogenicity in the human body is not a clear and concise technical feature.
Claim 5 is unclear since it refers to safety and costliness as features which are no technical features.
Claim 6 is unclear since it refers to undefined compounds and structures and does not clearly and concisely describe the usage of the antibody fragment of the application for "generating all kinds of possible molecules".
Claim 7 is unclear because it is directed towards a use "for developing a diagnostic reagent for the detection of hepatitis B surface antigen" which is not a clear and concise technical feature lacking definition and was therefore excluded from the search as well as the establishment for novelty, inventive step and industrial applicability.

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Roguska MA, Pedersen JT, Keddy CA, Henry AH, Searle SJ, Lambert JM, Goldmacher VS, Blättler WA, Rees AR, Guild BC. "Humanization of murine monoclonal antibodies through variable domain resurfacing." Proc Natl Acad Sci U S A. 1994 Feb 1;91(3):969-73. *Abstract, page 970 left column 2nd paragraph and right column 3rd paragraph, Figure 1, page 972, left column last paragraph* ----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/IN 2008/000796

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
A		none	