Article type: Original Article - Hepatology (Experimental)

Received date: 10-Aug-2013

Accepted date: 13-Nov-2013

Monoclonal antibodies to various epitopes of HBs antigen inhibit HBV infection

Forough Golsaz Shirazi[a], Hamed Mohammadi[a], Mohammad Mehdi Amiri[a], Katrin Singethan[b], Yuchen Xia[b], Ali Ahmad Bayat[c], Motahareh Bahadori[c], Hodjatallah Rabbani[c], Mahmood Jeddi –Tehrani[c], Ulrike Protzer[b], Fazel Shokri[a,c]

a) Department of Immunology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

b) Institute of Virology, Technische Universität München / Helmholtz Zentrum München, Munich, Germany

c) Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

Corresponding author:

Professor Fazel Shokri; Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, IRAN

Email: fshokri@tums.ac.ir

Tel: 0098 21 22432020

Fax: 0098 21 22432021

Running title: HBV neutralizing monoclonal antibodies

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/jgh.12483

This article is protected by copyright. All rights reserved.

Accepted Article

Abstract

Background and Aim

Antibodies against the “a” determinant of hepatitis B surface antigen (HBsAg) are able to neutralize circulating HBV particles and to prevent HBV infection. It has been proposed, that a single amino acid exchange may allow the virus to escape the immune response. We used a set of monoclonal antibodies (MAbs) to investigate whether a single mutation may account for virus escape from humoral immunity.

Methods

Nine murine HBsAg specific MAbs were raised. Reactivity of all antibodies with 14 recombinant mutants of HBsAg was assessed by ELISA. HBV infection of HepaRG cells was used to evaluate viral neutralization capacity of MAbs *in vitro*.

Results

All MAbs were able to inhibit the establishment of HBV infection in a dose dependent fashion, but recognition of HBsAg variants varied. The MAbs were classified into 3 subgroups based on their pattern of reactivity to the HBsAg variants. Accordingly, three MAbs showed weak reactivity (<40%) to variants with mutations within the first loop of "a" determinant, five MAbs displayed negligible binding to variants with mutations within the second loop and one MAb lost its binding to variants having mutations in both loops of the "a" determinant.

This article is protected by copyright. All rights reserved.

Accepted Article

Conclusions

Our results indicate that antibodies against different epitopes of the "a" determinant of HBsAg are able to neutralize HBV. It seems that mutations within a single or a limited number of amino acids within this determinant can hardly result in viral escape. These results have important implications for the development of antibody-based therapies against HBV.

Key Words: "a" determinant, HBsAg, HBV neutralization, Single amino acid mutation, Monoclonal antibodies, viral escape.

Introduction

Hepatitis B virus (HBV) causes acute and chronic liver infections in humans that can lead to development of liver cirrhosis and hepatocellular carcinoma (HCC) [1].

Recurrence of HBV infection in patients who undergo liver transplantation is a major problem which occurs at a rate of about 78-90% [2]. Frequency of recurrent HBV infection in patients who use high dose of HBIG for 3 years after transplantation, is reduced to 20-35% [3]. Long-term use of HBIG can provoke emergence of genetic HBV mutants, which may cause the virus to become resistant to neutralization [2].

In HBV infection, antibodies against epitopes in the external loop of HBsAg, the so called "a" determinant located within the major hydrophilic region of the surface antigen, are neutralizing and could contribute to clearance of circulating HBV particle [4].

Mutation rate of HBV is more than 10-fold higher than other DNA viruses, because the virus replicated via reverse transcription, which is error prone [5]. These mutations can induce HBsAg conformational changes, which can lead to false-negative results in HBsAg detection immunoassays [6].

Emergence of HBV escape mutants is due to immune pressure induced by vaccine, HBIG or natural infection [5, 7]. The predominant mutant is G145R, which is caused by substitution of a

This article is protected by copyright. All rights reserved.

Accepted Article

glycine to an arginine at position 145 of HBsAg. Depending on the HBV genotype, D144A is a second important mutant especially in HBIG treated patients [8]. Therapies such as interferon-α, lamivudine, entecavir and tenofovir, which are used currently as FDA approved treatments, have been shown to control HBV infection effectively, but do not eliminate the virus [9]. Since vaccination failure in preventing blood-born transmission from an infected mother to her newborn is almost 10%, development of combined therapy or new approaches for preventing HBV replication are essential [10].

The effect of HBsAg mutations on immunogenicity and antigenicity of this antigen is not still clear. Since "a" determinant is the major immunogenic region of HBsAg [11], it is necessary to determine whether antibodies against this region could still be protective after mutation in this region.

If the antibody response to various epitopes within this region could neutralize the virus, limited mutations within some of these epitopes, may hardly be able to induce escape mutants capable of evading the immune response.

We studied the reactivity of a panel of anti HBs MAbs, which are able to prevent wild type HBV infection, with mutant forms of HBsAg. Some of these MAbs failed to react with one or more mutant forms of HBsAg, but the combination of these MAbs seems to be able to compensate and complement each other and neutralize the mutant viruses. These data offer perspectives for interpretation of the real mechanism of HBV escape mutation and present new aspects for improving anti-HBs antibody based Immunoprophylaxis for hepatitis B.

This article is protected by copyright. All rights reserved.

Accepted Article

Methods and Materials

Production and Screening of Anti HBs monoclonal antibody secreting hybridomas

Eight weeks old Balb/C mice were immunized with 1ug of HBsAg, adw2 sybtype (Heberbiovac, Heberbiotec, S.A., Havana, Cuba) in Freund's complete adjuvant (Sigma, St. Louis, MO, USA), intraperitoneally (i.p.) to produce monoclonal antibodies, as described elsewhere [12].

Microtiter ELISA plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight with 1ug/ml HBsAg at +4°C. After blocking with 3% skim milk, supernatants of growing hybridomas were added to the wells and incubated for 1h and then HRP-conjugated goat anti mouse immunoglobulin (Sigma) was added. Following 1h incubation 3,3',5,5' Tetramethylbenzidine (TMB) substrate solution (PishtazTeb, Tehran, Iran) was added and the reaction was stopped by adding 1M HCL. Optical density (OD) was finally measured at 450/620 nm.

Isotype determination of MAbs by capture ELISA

ELISA plate was coated with goat anti-mouse IgG1, IgG2a, IgG2b and IgG3 (ISO-2 kit, Sigma). Supernatant of the hybridoma cells was added to the wells and incubated for 1h. After washing, appropriate dilution of HRP-conjugated goat anti mouse Ig was added. Following washing, the reaction was revealed with TMB substrate and the OD measured as described above.

This article is protected by copyright. All rights reserved.

Accepted Article

HiTrap column of streptococcal protein G (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to purify MAbs from ascites.

Accepted Article

This article is protected by copyright. All rights reserved.

Determination of affinity constant of MAbs

Wells of an ELISA plate were coated with 2.5, 1.2, 0.6, 0.3ug/ml of HBsAg. After blocking, purified antibodies were serially diluted and added to the wells and incubated for 1h at 37°C. Following washing, conjugated goat anti-mouse IgG was added. TMB substrate was added after washing and the absorbance was measured. Sigmoidal curves of ODs versus logarithmic antibody concentrations were plotted and final K_{aff} calculated using the equation described elsewhere [12-13].

RNA isolation, RT-PCR and VH-VL gene determination

For cDNA preparation, total RNA was extracted from 6×10^{6} hybridoma cells as described previously [14] .Two PCR reactions were run for each hybridoma cDNA, one for heavy chain and the other for light chain amplification. Primer sequences which were used to amplify heavy and light chains:

VH/degenerate sense primer: CAGGTSMARCTGCAGSAGTCWGG

VH anti-sense primer: AGGGGCCAGTGGATAGACAGATGG

Vk sense primers set: include 11 family specific primers [15].

V Kappa anti-sense primer: TGGTGGGAAGATGGATACAG

This article is protected by copyright. All rights reserved.

Accepted Article

SDS-PAGE and Western blot analyses

One microgram of HBsAg was run under reduced (8% of 2-mercaptoethanol) and non reduced conditions on 12% (wt/vol) polyacrylamide gel and transferred to PVDF membrane (Amersham Pharmacia, Glattbrugg, Switzerland). The blot was incubated with appropriate concentrations of purified anti-HBs MAbs. Subsequently, the membrane was incubated with HRP-conjugated goat anti mouse Ig. Finally the blots were developed with enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL USA).

Immunofluorescence assay

The human hepatoma HBV-positive cell line HepG2.2.15 and its parental HBV-negative cell line HepG2, were maintained in collagen-coated 8-well tissue culture chamber slides (Invitrogen, Karlsruhe, Germany) at 37°C and 5% CO2 until the cells reached approximately 60-80% confluency. Cells were fixed by 4% paraformaldehyde (PFA). Fixed cells were permeabilized with Triton X100 for 10 minutes and blocked with 10% goat serum. Permeabilized cells were incubated with 2.5ug/ml of MAbs overnight at 4°C. The same concentration of a murine MAb with irrelevant specificity was used as a negative control. The slides were washed in 0.1% Tween-20 PBS, and incubated with goat anti-mouse Ig fluorescein isothiocyanate (FITC) conjugate (Sigma-Aldrich) for 1h. Slides were washed, air-dried and covered using Fluoromount solution as a mountant.

This article is protected by copyright. All rights reserved.

Accepted Article

Immunoscreening of anti-HBs MAbs with mutant HBsAg

Purified MAbs were coated on ELISA plate at 5ug/ml concentration. Appropriate concentrations of commercial recombinant mutant HBsAg, adw2 subtype, including T143K, M133H, Q129L, T126N (GenWay Biotech Inc, San Diego, USA) and supernatant of cultured CHO cells which were transfected with vectors encoding mutant forms of HBsAg including P120E, T123N, Q129H, M133L, K141E, P142S, D144A, G145R, N146S and C147S (produced in our lab, unpublished data) were added to the solid phase. Afterwards, sheep anti-HBs biotinylated conjugate (PishtazTeb, Tehran, Iran) was added and incubated for 1h at 37°C. After extensive washing, streptavidin-HRP conjugate (Invitrogen) was added and incubated for 1h at 37°C. Subsequently, TMB substrate solution was added and OD measured. Supernatant of nontransfected CHO cells was included as negative control. The OD obtained for wt HBsAg reaction was taken as 100%. The signal obtained for each individual mutant at the preset concentration was compared with the wt signal and expressed as percent change of reactivity, based on Cooreman et al [16]. The concentration of these mutant forms in the supernatant of transfected CHO cells was measured with commercial ELISA kit (BioELISA HBsAg, Biokit, Barcelona, Spain) which employs polyclonal antibodies for both capture and detection layers and recognizes mutant forms of HBsAg with high specificity and sensitivity.

HepaRG differentiation

To induce HepaRG cell differentiation, a two-step procedure was used. HepaRG cells were cultured for 2 weeks in William's E medium (Invitrogen) supplemented with 10% fetal bovine serum, Penicillin/Streptomycin 50U/ml, 5ug/ml insulin and 5×10^{-5}M hydrocortisone

This article is protected by copyright. All rights reserved.

Accepted Article

hemisuccinate (all purchased from Invitrogen). Afterwards the medium was supplemented with 0.8% dimethyl sulfoxide (DMSO) (Sigma) for 2 additional weeks.
HepaRG cells were subsequently differentiated into morphologically and functionally mature hepatocyte-like cells [17].

Preparation of virus stock for infection of HepaRG cells

The human hepatoma cell line HepG2.2.15 which is capable of producing HBV was maintained on collagen-coated culture plates. Cells were cultured in DMEM supplemented with 10% FCS. Confluent HepG2.2.15 cells were then cultured in Williams E medium supplemented with 5% FCS plus other ingredients mentioned above and 2.4µg/ml hydrocortisone and 0.75% DMSO. Supernatant of the HBV producing HepG2.2.15 cell line was concentrated using Centricon Plus-70 (Biomax100; Millipore Corp., Billerica, MA). In order to measure the amount of HBV copy number in the virus stock, dot blotting was performed, using 50ul of concentrated HBV stock sedimented into a 1.15-g/ml to 1.44-g/ml CsCl step gradient covered with 0.5 ml of 20% sucrose by centrifugation at 55,000 rpm for 16h at 10°C in an SW 61 swing-out rotor (XL-70 ultracentrifuge; Beckman, USA). All the fractions were collected separately and subjected onto a positively charged nylon membrane. HBV DNA was detected by hybridization with a 32 P-labeled HBV DNA probe (Hartmann Analytics, Braunschweig, GmbH) as described elsewhere [18].

HBV infection neutralization by anti HBs MAbs

Different concentrations of anti HBs MAbs (10, 2.5, 1, 0.6, 0.2 and 0.05ug/ml) were preincubated with HBV preparation (MOI (multiplicity of infection) =200) for 2.5h at 37°C.

This article is protected by copyright. All rights reserved.

Accepted Article

Infection of differentiated HepaRG cells was performed in medium containing 4% PEG 8000 (Sigma). After overnight incubation, cells were washed 4 times with PBS and cultured for 12 days as described above. Supernatant was collected 3 times at days 4, 8 and 12. On day 12 total DNA was extracted. As negative and positive controls, mouse monoclonal IgG1 (Abcam, Cambridge, MA, USA) and 0.1IU of HBIG (Hepatec, Biotest Pharma, Dreieich, Germany) were applied, respectively.

Assays for detecting HBV infection

The establishment of HBV infection was determined by measuring nuclear HBV cccDNA (covalently closed circular DNA) and HBe Ag secretion from infected cells.

Microspin columns (Macherey-Nagel, Dueren, Germany) were used to extract total DNA from infected HepaRG cells. In order to quantify HBV cccDNA, real-time PCR was performed using LightCycler Fast Start DNA Master Plus SYBR green I mix (Roche Diagnostics, Mannheim, Germany) with specific primers. The genes were normalized to PrP gene (Prion Protein, as internal control). The primers which were used to amplify cccDNA and Prp, respectively [19-20] were as follows:

HBVccc-forward: GCCTATTGATTGGAAAGTATGT, HBVccc-reverse: AGCTGAGGCGGTATCTA

PRP-forward: TGCTGGGAAGTGCCATGAG, PRP-reverse: CGGTGCATGTTTTCACGATAGTA

The amount of HBe antigen in the supernatant of infected cells was measured using a commercial BEPIII ELISA kit (Siemens Diagnostic, Marburg, Germany).

The percent inhibition for each MAb was calculated as follow:

This article is protected by copyright. All rights reserved.

Accepted Article

%inhibition = ((Average value of triplicate measurements for each test)-(Average value of triplicate measurements for the corresponding positive control)/ (Average value of triplicate measurements for the corresponding positive control) ×100.

Where the test is HepaRG cells treated with HBV and anti HBs MAb and positive control represents HepaRG cells treated with HBV in presence of an irrelevant mAb of the IgG1 isotype.

Accepted Article

This article is protected by copyright. All rights reserved.

Results

Production of anti-HBs monoclonal antibodies

Nine anti HBs MAbs which could recognize wt HBsAg were generated. All MAbs were purified from ascitic fluids by protein G column. Purified MAbs were then characterized for isotype, affinity and specificity. The results are outlined in table 1.

Amplification and Cloning of immunoglobulin variable region heavy (VH) and light (VL) chain genes

The VH and VL genes were amplified from cDNA of hybridoma clones and cloned into the TA cloning vector. The cloned VH and VK genes were then sequenced. Different combinations of VH, DH, JH, VK and JK genes were expressed in each clone indicating distinct clonal origin (Table 1).

Specificity determination of MAbs by immunoblotting

The HBsAg specificity of each of the MAbs was determined by Western blot analysis. Representative results obtained for some of the MAbs are shown in figure 1. The results showed that all MAbs could recognize both reduced and non-reduced forms of HBsAg. The non-reduced form of HBsAg was composed of extensively polymerised antigen and a small amount of dimer antigen. The polymerised antigen neither entered the gel nor transferred to PVDF membrane due to its large size. Upon reduction, the polymerised antigen was reduced

This article is protected by copyright. All rights reserved.

Accepted Article

to monomer, dimer and trimer bands (Figure 1A). Other studies have also confirmed aggregation of HBsAg [21-22].

Accepted Article

This article is protected by copyright. All rights reserved.

Immunoreactivity of anti-HBs MAbs with mutant forms of HBsAg

Immunoreactivity of anti-HBs MAbs was assessed by ELISA on 14 recombinant HBsAg mutants including T143K, M133H, T126N, Q129L P120E, T123N, Q129H, M133L, K141E, P142S, D144A, G145R, N146S and C147S as well as wt HBsAg. All antibodies reacted with wt HBsAg, but some MAbs either reacted weakly or failed to bind to some mutant antigens. Accordingly, MAbs were classified based on their pattern of reactivity with HBsAg variants having mutations within either the first or the second loop of "a" determinant, into three groups. The first group comprises 5G7, 3C9 and 2F2 antibodies shows either reduced or no reactivity with T126N and Q129L mutants. The second group (1C10, 4G4, 3A7, 2F1 and 6E3 MAbs) was able to recognize T126N and Q129L, but displayed low or no reactivity to K141E, T143K, D144A, G145R and/or C147S variants. The third group of MAb (S4A) showed a mixed pattern of reactivity (Table 2).

Immunofluorscence staining of HepG2.2.15 cells by anti-HBs MAbs

Four MAbs (2F2, 6E3, 2F1 and 3A7) were employed to determine their pattern of reactivity with HepG2.2.15 cells by immunofluorscence staining. A diffuse granular intracellular and membrane labelling was observed in HepG2.2.15 cells, which is interpreted by endoplasmic reticulum translation of HBsAg and its transport to the cell membrane via Golgi apparatus (Figure 2).

Immunofluorscence staining was also performed with HepG2 cells as HBV-negative counterpart of HepG2.2.15 cells which showed no positive staining (data not shown).

This article is protected by copyright. All rights reserved.

Accepted Article

Neutralization of HBV infection by anti-HBs MAbs in HepaRG cells

Concentrated HBV particles pretreated with different concentrations (10, 2.5 and 0.6ug/ml) of MAbs, were incubated with differentiated HepaRG cells. Establishment of HBV infection was determined by detection of HBe antigen in supernantant and HBV cccDNA in lysate of HepaRG cells. The MAbs were classified according to their inhibitory effect on HBV infection biomarkers (HBeAg and cccDNA) in HepaRG cells. MAbs 2F2, 1C10 and 6E3 were able to almost completely inhibit HBV infection in HepaRG cells even at the lowest concentration (0.6ug/ml) employed in this study (Figure 3A). These MAbs were also used at lower concentrations (0.2 and 0.05ug/ml) to evaluate whether they are still capable of inhibiting the virus infection. Our results demonstrated that they can still induce 40-90% inhibition at 50ng/ml. The remaining MAbs including 3C9, 2F1, S4A, 5G7, 3A7 and 4G4, inhibited HBV infection dose-dependently, but to a lesser extent than the first group (Figure 3B). Application of 600ng/ml of these MAbs induced 5-75% viral neutralization.

Discussion

In the present study we demonstrated that MAbs specific for various epitopes within the "a" determinant of HBsAg were able to neutralize HBV infection in vitro using the human HepaRG. The reactivity of nine MAbs with 12 variants having single mutation inside the "a" determinant as well as two variants with mutations at positions 120 and 123 was assessed. P120E and T123N were included in our HBsAg mutant panel due to their proximity to cysteine residues 121 and 124 and their impact on formation of disulphide bridge [23]. There are five cysteine residues within the "a" determinant of HBsAg located at positions 121, 124, 137, 139 and 147 which could form two disulfide bonds leading to formation of at least two

This article is protected by copyright. All rights reserved.

Accepted Article

distinct loops [24-25]. The proximal loop is located within cysteine residues 124 and 137 and the distal loop constitutes amino acids 139-147. Our MAbs were found to recognize epitopes located within either or both loops. Accordingly, MAbs were classified based on their pattern of reactivity with mutant forms of HBsAg into three groups. The first group includes 5G7, 3C9 and 2F2 antibodies which show either reduced or no reactivity with epitopes inside the proximal loop mutated at positions 126 (T126N) and/or 129 (Q129L) residues. The second group (1C10, 4G4, 3A7, 2F1 and 6E3 MAbs) was able to recognize T126N and Q129L, but displayed low or no reactivity to variants mutated at positions within the distal loop including K141E, T143K, D144A, G145R and/or C147S. The third group of MAbs (S4A) showed a mixed pattern of reactivity (Table 2). The latter group of MAbs seems to recognize epitopes sharing amino acids from both loops or epitopes from both loops located in proximity to each other. Many of our MAbs couldn't recognize or showed reduced reactivity to T123N and G145R mutants, suggesting importance of these residues in immunogenicity of HBsAg [8, 24, 26]. Assignment of MAb specificity to the first or second loop of HBsAg is not absolute, taking in to consideration the results obtained from the mutant HBsAg panel. Obviously, mutations within either the first or second loop of the "a" determinant might affect the conformation of the whole determinant leading to reduced binding of some MAbs to their target epitopes located in the opposite loop. For instance, IC10, 6E3 and 2F1 MAbs which have been assigned to the second loop of “a” determinant based on their low or lack of reactivity with second loop mutants, displayed to a lesser extent reduced reactivity to the first loop mutants including Q129L and M133L. The reduced reactivity of these MAbs to Q129L and M133L is most likely associated to conformational changes induced by these two mutations on the target epitopes recognized by these MAbs within the second loop. The so called fit-induced conformational changes might influence a variety of epitopes located in the vicinity of the target epitope.

This article is protected by copyright. All rights reserved.

Accepted Article

Increasing concentrations of some MAbs and their cumulative effect were checked to assess their influence on enhancement of reactivity of these MAbs with the negative mutant forms of HBsAg. The results (data not presented) showed in some cases, increasing concentration of MAb marginally improves their reactivity with the target mutation. However, using a combination of MAbs, didn't show any difference in the results. Taken together, increasing concentration of MAbs didn't show a significant effect on reactivity pattern of MAbs with mutant forms of HBsAgs.

Interestingly, all MAbs regardless of their pattern of reactivity were able to inhibit HBV infection and replication efficiently, as evidenced by the dose dependent reduction of HBeAg secretion and cccDNA synthesis in HBV treated HepaRG cells. Some of these MAbs could neutralize the virus even at 0.05ug/ml concentration around 90% (6E3) and 50% (1C10, 2F2). We couldn’t find any correlation between antibodies affinity and their pattern of reactivity with mutant forms of HBsAg or viral neutralization capacity. In fact, antibody affinity is an important factor in virus neutralization capacity of MAbs, but isn't considered as the only factor. Antibody specificity is another crucial parameter which may influence HBV neutralization. Some MAbs may have low affinity against HBsAg, but they could neutralize the virus efficiently because they bind to epitopes located inside or in the vicinity of the conserved area that virus use to attach to hepatocytes. However, MAbs which recognize epitopes away from this area could bind to the virus with a higher affinity, but at lower neutralization efficiency.

The fact that all MAbs displayed different patterns of reactivity with the mutant panel of HBsAgs and expressed different combinations of VH, DH, JH, VK and JK genes indicate that they have distinct entity and were derived from different B cell clones (Table 1).

Clearance of HBV requires the coordinated interaction of innate and adaptive humoral and cellular immune responses [27].

This article is protected by copyright. All rights reserved.

Accepted Article

Antibodies against the "a" determinant are thought to be pivotal for HBV clearance and could neutralize the major HBV subtypes. Therefore the "a" determinant is a crucial target for diagnosis and immunoprophylaxis [16]. It is assumed that mutation changes immunological specificities of S protein and could lead to propagation of the mutant forms of the virus in the presence of neutralizing antibodies [16].

An amino acid substitution within the "a" determinant, however, neither necessarily impairs the immunogenicity of HBsAg, nor reduces the binding of anti-HBs antibodies. Zheng et al have shown that HBsAg G145R is immunogenic and still able to induce mutant-specific antibody response in mice. Mutation within the "a" determinant like G145R probably results in a significant conformational change, which doesn't lead to loss of immunogenicity, but instead creates a new specificity [11].

Our results are consistent with this assumption. We generated a variety of MAbs with specificities for different epitopes within the "a" determinant of HBsAg. All these antibodies efficiently inhibited HBV replication in HepaRG cells indicating that variation within a single epitope or a limited number of epitopes of HBsAg may not necessarily lead to emergence of an escape mutant capable of evading the humoral immune response. Hepatitis B variants such as T126N [28-29], Q129L [11, 30], Q129H [28], P142S [28-29], T123N [31], D144A [28-29], K141E [32], M133L and G145R [33] have been identified as immune escape mutants. Indeed, some of our MAbs were able to recognize the mutant HBsAg epitopes which have been reported to lead to the emergence of vaccine or HBIG induced escape mutants. All our MAbs, were able to strongly neutralize the wild type virus. This suggests that selection of a single amino acid exchange does not abolish the protective antibody response against other immunoprotective epitopes of HBsAg.

In patients dominantly carrying a mutant variant of the virus, there is a possibility that none of the immune mechanisms works properly, or the frequency of HBV specific T cells is too

This article is protected by copyright. All rights reserved.

Accepted Article

low or these specific T cells recognize different subdominant HBV epitopes. Lack of protective antibody response has also been reported in normal individuals vaccinated with highly immunogenic recombinant HBsAg [34]. This was associated with lower frequency of HBsAg-specific B cell repertoire [35-36] or a shift in the Th1/Th2 response [37], suggesting that other reasons besides the emergence of HBV variants may be responsible for immune evasion.

A panel of HBV-neutralizing MAbs which are capable of recognizing multiple epitopes of the "a" determinant, even in the absence of antibodies specific for escape mutant forms of this determinant, will be beneficial in immunoprophylaxis of HBV infection as an immunotherapeutic agent. It can serve for prolonged therapy with fewer adverse effects. Individuals who are exposed to HBV-positive material (needle stick or cut injury), newborns of mothers who are HBV carriers and patients who undergo liver transplantation are candidates for such therapy [38].

Our results could provide the insight that the immune system produces a large number of antibodies against a variety of epitopes within the "a" determinant. Although some antibodies may lose their ability to recognize the virus after mutation, there is still a big enough repertoires of specific antibodies, which could recognize other conserved epitopes and neutralize the virus variant. Further studies is necessary to clarify whether these MAbs are able to neutralize the mutant variants of the virus and inhibit HepaRG infection.

Acknowledgements

We thank Theresa Asen, Romina Bester, Kerstin Ackermann for their technical support, Dr Julie Lucifora, Dr Mohammad Hodjat Farsangi, Beate Schittl and Xiaming Cheng for

This article is protected by copyright. All rights reserved.

Accepted Article

scientific consultation and Dr Boutorabi and Pishtaz Teb Diagnostics for providing anti HBsAg conjugate.

Funding

This study was supported in part by grants from Avicenna Research Institute and Iran National Science Foundation.

Competing interests

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article

References

[1] Chen Y, Sze J, He ML. HBV cccDNA in patients' sera as an indicator for HBV reactivation and an early signal of liver damage. *World J Gastroenterol*. 2004; 10: 82-5.

[2] Katz L, Paul M, Guy D, Tur- Kaspa R. Prevention of recurrent hepatitis B virus infection after liver transplantation: hepatitis B immunoglobulin, antiviral drugs, or both? Systematic review and meta- analysis. *Tranpl Infect Dis*. 2010; 12: 292-308.

[3] Marzano A, Salizzoni M, Debernardi-Venon W, *et al.* Prevention of hepatitis B virus recurrence after liver transplantation in cirrhotic patients treated with lamivudine and passive immunoprophylaxis. *J Hepatol*. 2001; 34: 903-10.

[4] Ogura Y, Kurosaki M, Asahina Y, Enomoto N, Marumo F, Sato C. Prevalence and significance of naturally occurring mutations in the surface and polymerase genes of hepatitis B virus. *J Infect Dis*. 1999; 180: 1444-51.

[5] Weber B. Genetic variability of the S gene of hepatitis B virus: clinical and diagnostic impact. *J Clin Virol*. 2005; 32: 102-12.

[6] Ly TD, Servant-Delmas A, Bagot S, *et al.* Sensitivities of four new commercial hepatitis B virus surface antigen (HBsAg) assays in detection of HBsAg mutant forms. *J Clin Microbiol*. 2006; 44: 2321-6.

[7] Aono J, Yotsuyanagi H, Miyoshi H, *et al.* Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis. *Hepatol Res*. 2007; 37: 731-9.

[8] Protzer- Knolle U, Naumann U, Bartenschlager R, *et al.* Hepatitis B virus with antigenically altered hepatitis B surface antigen is selected by high- dose hepatitis B immune globulin after liver transplantation. *Hepatology*. 2003; 27: 254-63.

[9] Panjaworayan N, Payungporn S, Poovorawan Y, Brown CM. Identification of an effective siRNA target site and functional regulatory elements, within the hepatitis B virus posttranscriptional regulatory element. *Virol J*. 2010; 7: 216.

[10] Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev*. 2000; 64: 51-68.

[11] Zheng X, Weinberger KM, Gehrke R, *et al.* Mutant hepatitis B virus surface antigens (HBsAg) are immunogenic but may have a changed specificity. *Virology*. 2004; 329: 454-64.

This article is protected by copyright. All rights reserved.

[12] Kazemi T, Tahmasebi F, Bayat AA, *et al.* Characterization of Novel Murine Monoclonal Antibodies Directed Against the Extracellular Domain of Human HER2 Tyrosine Kinase Receptor. *Hybridoma*. 2011; 30: 347-53.
[13] Hajighasemi F, Saboor-Yaraghi AA, Shokri F. Measurement of affinity constant of anti-human IgG monoclonal antibodies by an Elisa-based method. *Iranian J Immunol*. 2004; 1: 154-61.
[14] Amiri MM, Jeddi-Tehrani M, Kazemi T, *et al.* Construction and characterization of a new chimeric antibody against HER2. *Immunotherapy*. 2013; 5: 703-15.
[15] Kontermann RE, Dèubel S. *Antibody Engineering*. New York: Springer, 2010.
[16] Cooreman MP, Van Roosmalen MH, Te Morsche R, *et al.* Characterization of the reactivity pattern of murine monoclonal antibodies against wild- type hepatitis B surface antigen to g145r and other naturally occurring "a" loop escape mutations. *Hepatology*. 1999; 30: 1287-92.
[17] Parent R, Beretta L. Translational control plays a prominent role in the hepatocytic differentiation of HepaRG liver progenitor cells. *Genome biology*. 2008; 9: R19.
[18] Arzberger S, Hösel M, Protzer U. Apoptosis of hepatitis B virus-infected hepatocytes prevents release of infectious virus. *J Virol*. 2010; 84: 11994-2001.
[19] Untergasser A, Zedler U, Langenkamp A, *et al.* Dendritic cells take up viral antigens but do not support the early steps of hepatitis B virus infection. *Hepatology*. 2006; 43: 539-47.
[20] Lucifora J, Arzberger S, Durantel D, *et al.* Hepatitis B virus X protein is essential to initiate and maintain virus replication after infection. *J Hepatol*. 2011; 55: 996-1003.
[21] Tleugabulova D, Falcón V, Sewer M, Pentón E. Aggregation of recombinant hepatitis B surface antigen in Pichia pastoris. *J Chromatogr B Biomed Sci Appl*. 1998; 716: 209-19.
[22] Tleugabulova D. Size-exclusion chromatographic study of the reduction of recombinant hepatitis B surface antigen. *J Chromatogr B Biomed Sci Appl*. 1998; 713: 401-7.
[23] Ijaz S, Ferns RB, Tedder RS. A 'first loop'linear epitope accessible on native hepatitis B surface antigen that persists in the face of 'second loop'immune escape. *J Gen Virol*. 2003; 84: 269-75.
[24] Tian Y, Xu Y, Zhang Z, *et al.* The amino acid residues at positions 120 to 123 are crucial for the antigenicity of hepatitis B surface antigen. *J Clin Microbiol*. 2007; 45: 2971-8.
[25] Alavian SM, Carman WF, Jazayeri SM. HBsAg variants: diagnostic-escape and diagnostic dilemma. *J Clin Virol*. 2012.
[26] Carman W. The clinical significance of surface antigen variants of hepatitis B virus. *J Viral Hepat*. 1997; 4: 11-20.

This article is protected by copyright. All rights reserved.

Accepted Article

[27] Rehermann B. Intrahepatic T cells in hepatitis B viral control versus liver cell injury. *J Exp Med*. 2000; 191: 1263-8.

[28] Caligiuri P, Sticchi L, Cerruti R, Icardi G, Bruzzone B. Presence of HBV S-gene mutants in immunocompromised patients. *New Microbiol*. 2012; 35: 97.

[29] Torresi J. Hepatitis B antiviral resistance and vaccine escape: two sides of the same coin. *Antivir Ther*. 2008; 13: 337.

[30] Blaich A, Manz M, Dumoulin A, *et al.* Reactivation of hepatitis B virus with mutated hepatitis B surface antigen in a liver transplant recipient receiving a graft from an antibody to hepatitis B surface antigen- and antibody to hepatitis B core antigen-positive donor. *Transfusion (Paris)*. 2012; 52: 1999-2006.

[31] Ie SI, Thedja MD, Roni M, Muljono DH. Prediction of conformational changes by single mutation in the hepatitis B virus surface antigen (HBsAg) identified in HBsAg-negative blood donors. *Virol J*. 2010; 7: 326.

[32] Peng X-M, Gu L, Chen X-J, Li J-G, Huang Y-S, Gao Z-L. Optimization of competitively differentiated polymerase chain reaction in detection of HBV basal core promoter mutation. *World J Gastroenterol*. 2005; 11: 3614.

[33] Lada O, Benhamou Y, Poynard T, Thibault V. Coexistence of hepatitis B surface antigen (HBs Ag) and anti-HBs antibodies in chronic hepatitis B virus carriers: influence of "a" determinant variants. *J Virol*. 2006; 80: 2968-75.

[34] Jafarzadeh A, Khoshnoodi J, Ghorbani S, Hazrati SM, Faraj Mazaheri B, Shokri F. Differential immunogenicity of a recombinant hepatitis B vaccine in Iranian neonates: influence of ethnicity and environmental factors. *Iran J Immunol*. 2004; 1: 98-104.

[35] Shokrgozar MA, Shokri F. Enumeration of hepatitis B surface antigen- specific B lymphocytes in responder and non- responder normal individuals vaccinated with recombinant hepatitis B surface antigen. *Immunology*. 2008; 104: 75-9.

[36] Shokrgozar M, Sam M, Amirkhani A, Shokri F. Frequency Analysis of HBsAg- specific B Lymphocytes in High- responder Individuals to Recombinant Hepatitis B Vaccine: Comparison of LDA and ELISPOT Assays. *Scand J Immunol*. 2006; 64: 536-43.

[37] Jafarzadeh A, Kardar GA, Khoshnoodi J, Shokri F. Downregulation of IL-12 production in healthy non-responder neonates to recombinant hepatitis B vaccine. *Iran Biomed J*. 2004; 8: 41-5.

[38] Eren R, Ilan E, Nussbaum O, *et al.* Preclinical Evaluation of Two Human Anti–Hepatitis B Virus (HBV) Monoclonal Antibodies in the HBV- Trimera Mouse Model and in HBV Chronic Carrier Chimpanzees. *Hepatology*. 2000; 32: 588-96.

This article is protected by copyright. All rights reserved.

Accepted Article

Table 1: Characteristics of anti HBs monoclonal antibodies

Light Chain		Heavy Chain			Isotype	Kaff (M^{-1})	Clone
JL	VL	JH	DH	VH			
IGKJ2*01	IGKV10-94*01	IGHJ1*01	ND	IGHV1S137*01	IgG2a	5×10^7	5G7
IGKJ2*01	IGKV10-94*01	IGHJ2*01	ND	IGHV1S81*02	IgG1	1.7×10^8	1C10
IGKJ2*01	IGKV10-96*01	IGHJ2*01	IGHD3-3*01	IGHV1-59*01	IgG2b	1.2×10^9	4G4
IGKJ2*01	IGKV4-72*01	IGHJ1*01	ND	IGHV1S137*01	IgG1	1.8×10^9	3C9
IGKJ5*01	IGKV4-59*01	IGHJ1*01	IGHD1-1*02	IGHV1-67*01	IgG1	1×10^9	3A7
IGKJ2*01	IGKV6-17*01	IGHJ2*01	IGHD2-1*01	IGHV5-12-1*01	IgG1	5×10^8	2F2
IGKJ4*01	IGKV1-117*01	IGHJ1*01	IGHD2-12*01	IGHV1-12*01	IgG2a	1.8×10^9	2F1
IGKJ2*01	IGKV12-41*01	IGHJ4*01	IGHD5-2*01	IGHV3-1*02	IgG2a	1.2×10^9	6E3
IGKJ2*01	IGKV1-110*01	IGHJ2*01	IGHD4-1*01	IGHV1S22*01	IgG1	3.3×10^8	S4A

ND: Not defined

This article is protected by copyright. All rights reserved.

Accepted Article

Accepted Article

Table 2: Immunoreactivity of anti HBs monoclonal antibodies with mutant forms of HBsAg

C147S	N146S	G145R	D144A	T143K	P142S	K141E	M133H	M133L	Q129H	Q129L	T126N	T123N	P120E	MAbs
++	+	+	+	+	+	++	++	+	++	-	-	-	+	5G7
+	+	+	-	+++	+	++	++	+	++	+	++	-	+	1C10
-	+	-	+	+++	+	+	++	+	++	++	+++	-	+	4G4
+	+	+	+	++	+	++	++	+	++	-	-	-	+	3C9
+	+	-	+	++	+	++	+	+	++	+	++	-	+	3A7
+	+	+	++	++	+	++	++	+	++	-	-	-	+	2F2
+	+	-	-	-	+	-	++	+	++	+	++	+	+	2F1
+	+	-	+	+++	+	++	++	+	++	+	++	-	+	6E3
-	+	-	-	++	+	+	-	++	++	+	++	-	-	S4A

Results are expressed as the ratio of OD obtained for reactivity of each MAb with mutant HBsAg to that of the wt HBsAg and presented as:
- :< 40%, +:40-80%, ++:80-120%, +++:>120. Wt: wild type HBsAg

This article is protected by copyright. All rights reserved.

Legends to figures:

Figure 1: Representative immunoblotting profile of some.

MAbs with reduced (A) and non reduced (B) HBsAg.

1: 4G4, 2: 5F9, 3: 1C10, 4: 3A7

Figure 2: Immunofluorescent labelling of HepG2.2.15 cells with anti-HBs monoclonal antibodies.

Reactivity of 2.5ug/ml of 4 anti-HBs antibodies (A: 2F1, B: 3A7, C: 6E3 and D: 2F2) was assessed with HepG2.2.15 cells. A murine MAb (2.5ug/ml) with irrelevant specificity used for negative control (data not presented). The fluorescence intensity of negative control was subtracted from each sample intensity.

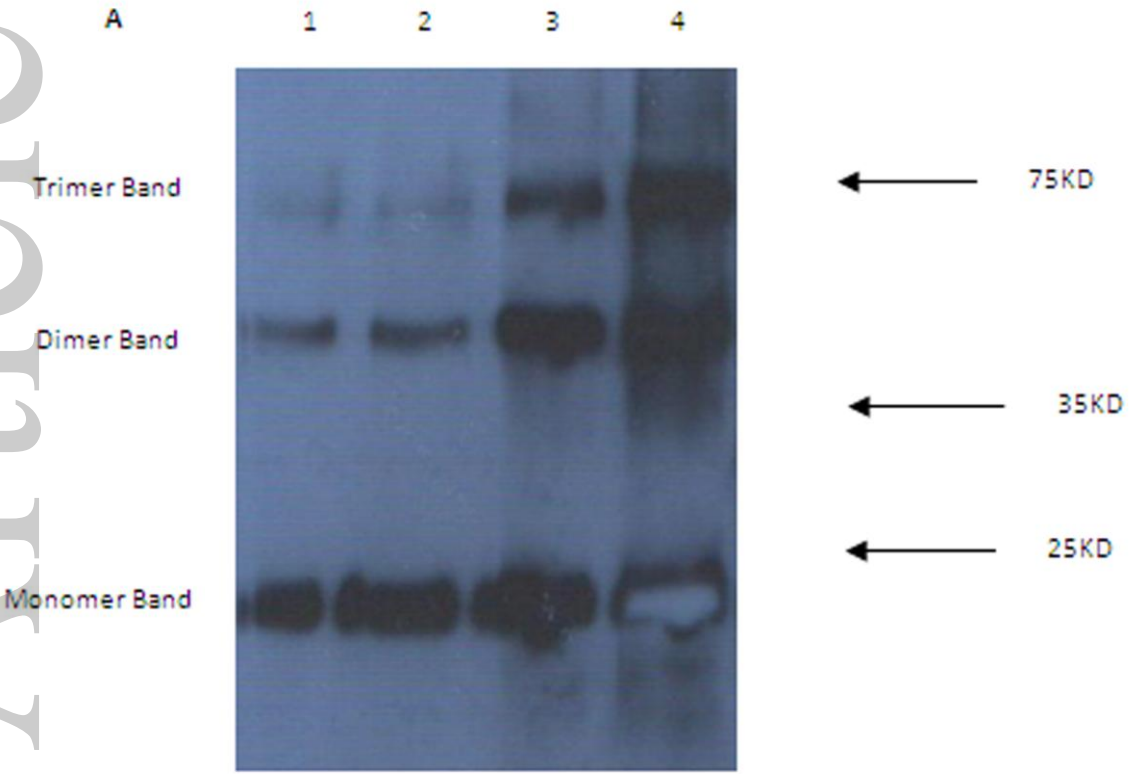
Figure 3: Inhibitory effect of anti-HBs monoclonal antibodies on HBV infection in HepaRG cells.

The MAbs were applied at either 10, 2.5, 1, 0.6, 0.2 and 0.05 ug/ml (A) or 10, 2.5 and 0.6 ug/ml (B) to concentrated HBV virus stock. HBe Ag (▬) and cccDNA (▨) were subsequently detected as infection biomarkers. A commercial HBIG preparation (Hepa) was used at 0.1 IU/ml as a positive control. This dataset is representative of results obtained in three independent experiments. The percent inhibition of each MAb was calculated based on the formula given in the Materials and Methods.

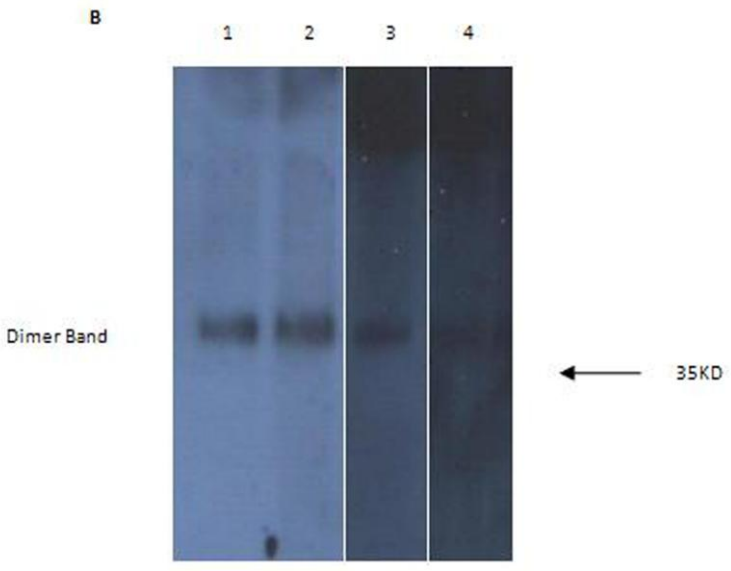
Accepted Article

This article is protected by copyright. All rights reserved.

Accepted Article



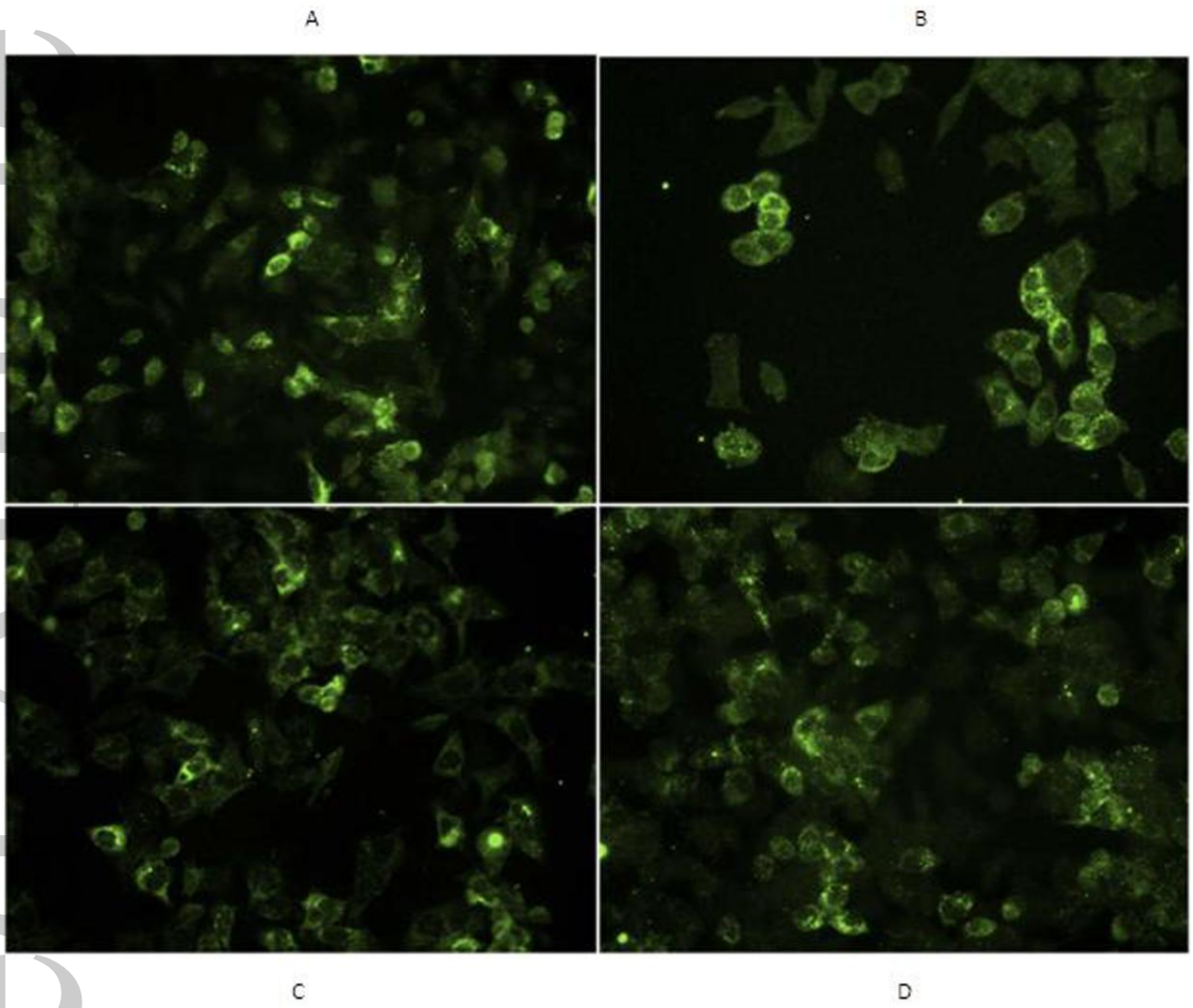
jgh_12483_f1A



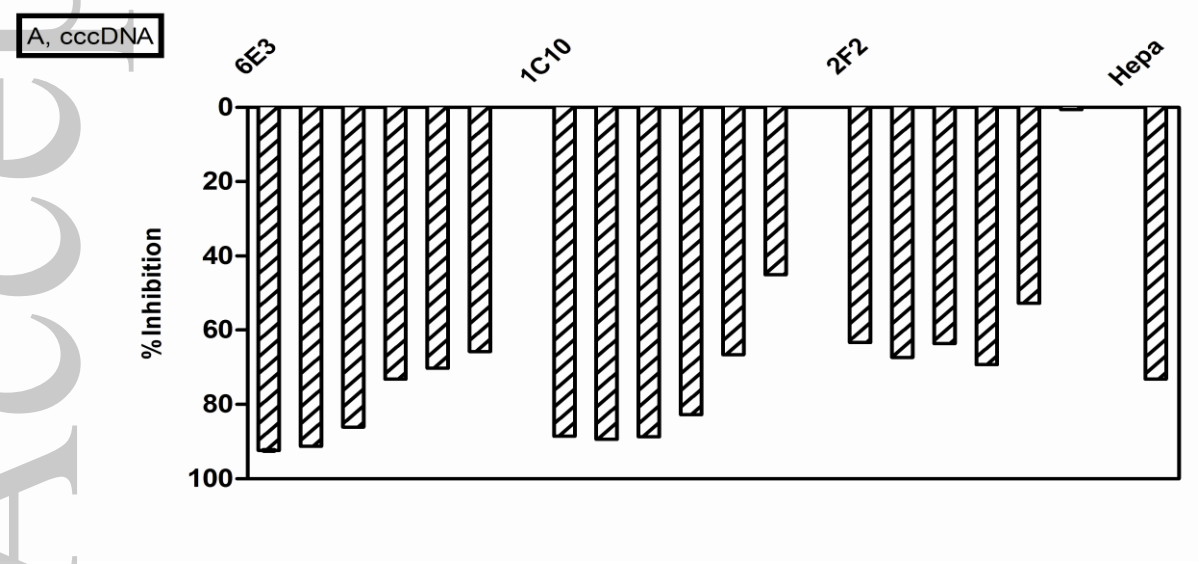
jgh_12483_f1B

This article is protected by copyright. All rights reserved.

Accepted Article



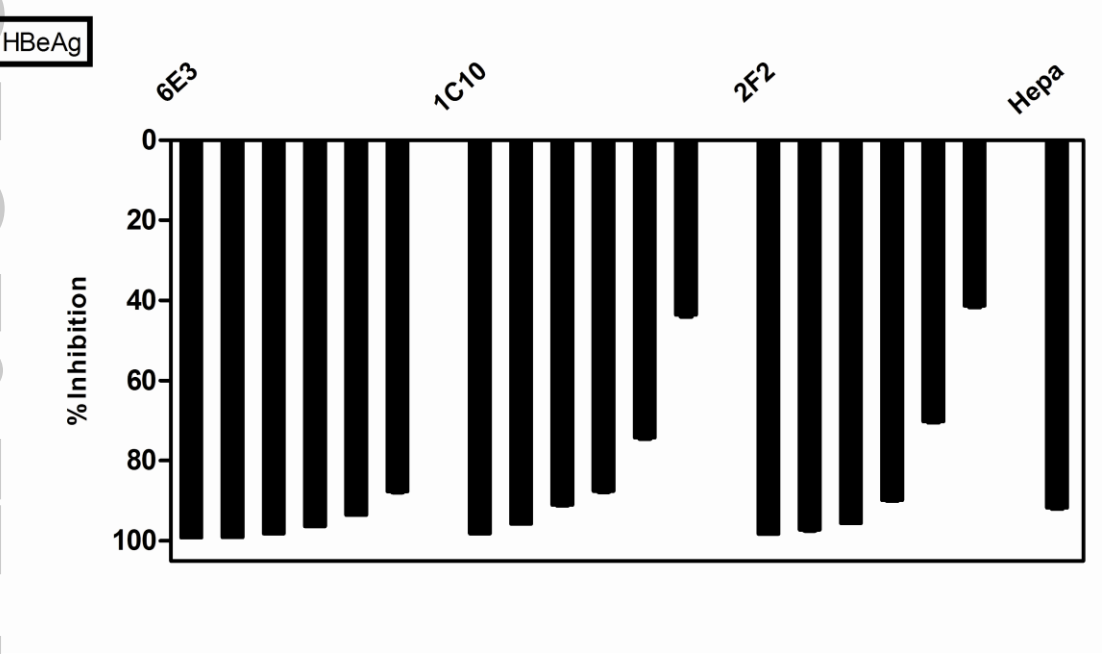
jgh_12483_f2



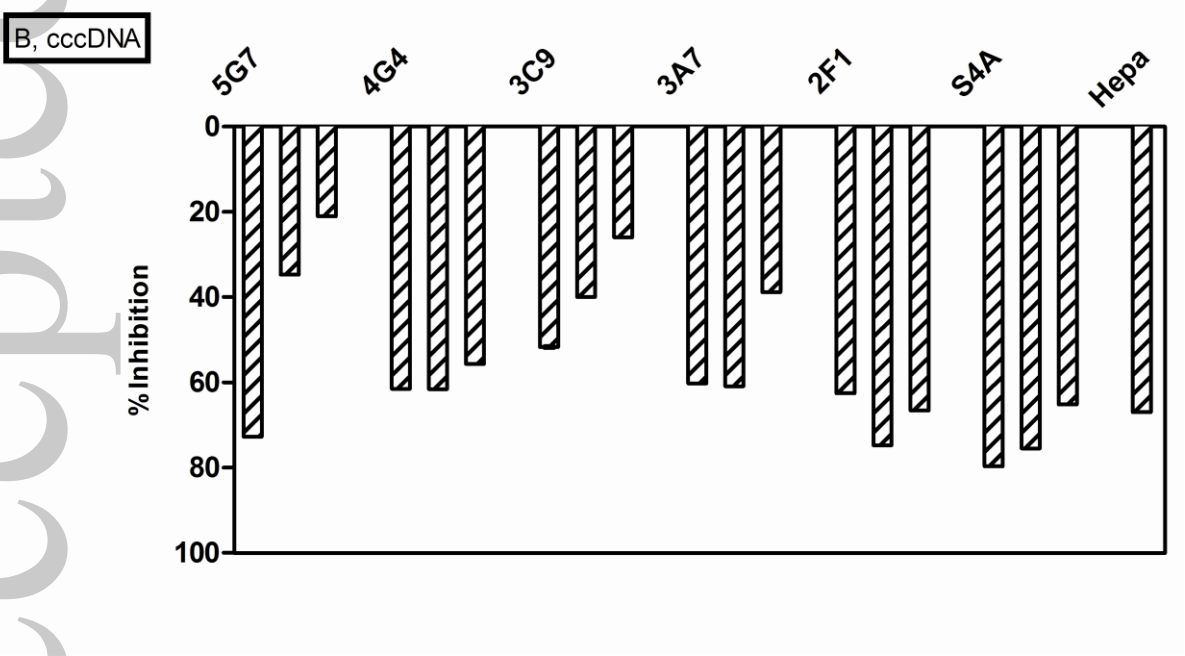
jgh_12483_f3AcccDNA

This article is protected by copyright. All rights reserved.

Accepted Article

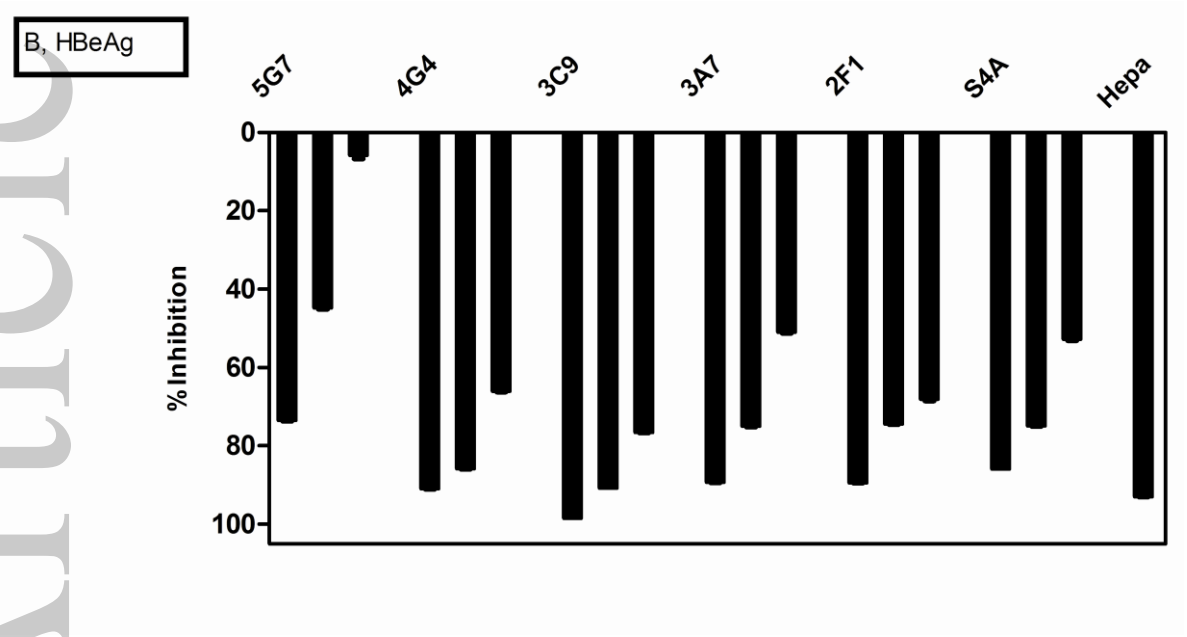


jgh_12483_f3AHBeAg



jgh_12483_f3BcccDNA

This article is protected by copyright. All rights reserved.



jgh_12483_f3BHBeAg

Accepted Article

This article is protected by copyright. All rights reserved.