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# Human monoclonal antibodies specific to hepatitis B virus generated in a human/mouse radiation chimera: the Trimera system

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## SUMMARY

An approach to develop fully human monoclonal antibodies in a human/mouse radiation chimera, the Trimera system, is described. In this system, functional human lymphocytes are engrafted in normal strains of mice which are rendered immuno-incompetent by lethal total body irradiation followed by radioprotection with severe combined immunodeficient (SCID) mouse bone marrow. Following transplantation, human lymphocytes colonize murine lymphatic organs and secrete human immunoglobulins. We have established this system as a tool to develop fully human monoclonal antibodies, and applied it for the generation of monoclonal antibodies specific for hepatitis B virus surface antigen. A strong memory response to hepatitis B surface antigen was elicited in Trimera engrafted with lymphocytes from human donors positive for antibodies to hepatitis B surface antigen. The human specific antibody fraction in the Trimera was $10^2$–$10^3$-fold higher as compared with that found in the donors. Spleens were harvested from Trimera mice showing high specific-antibody titres and cells were fused to a human–mouse heteromyeloma fusion partner. Several stable hybridoma clones were isolated and characterized. These hybridomas produce high-affinity, IgG, anti-hepatitis B surface antigen antibodies demonstrating the potential of the Trimera system for generating fully human monoclonal antibodies. The biological function and the neutralizing activity of these antibodies are currently being tested.

## INTRODUCTION

Antibodies have been shown to neutralize micro-organisms, destroy cancer cells and modulate the immune system.[1] Thus, antibody therapy can have great potential value for treatment of cancer, autoimmune disorders and viral or bacterial infections. Monoclonal antibodies (mAb) from mouse origin are relatively easy to produce but their therapeutic utility is restricted by their immunogenicity in patients, causing human anti-mouse antibody (HAMA) response.[2] Monoclonal antibodies from human origin may function better therapeutically, however, these are difficult to raise. The most traditional method by which to generate human mAb involves fusion of human lymphocytes from pre-immunized donors, usually after Epstein–Barr virus (EBV) stimulation, to a fusion partner to obtain hybridoma clones.[3,4] Alternative methods include genetic modification of murine mAb to create chimeric[5] and humanized[6] antibodies which consist of the mouse binding sites combined with human antibody elements. However, the resulting antibodies retain murine sequences that render them significantly more immunogenic than human antibodies,[2] thus preventing successful application of these antibodies in therapy *in vivo*. Another approach to generate human mAb is the use of human variable regions phage display libraries that can be screened for $V_H$ and $V_L$ combinations of the right specificity.[7,8] The transgenic mouse approach make use of mice carrying human V-gene segments in germ-line configuration.[9] Recently, human mAb were developed in transgenic mice that express human IgM, IgG, and Igκ in the absence of the mouse IgM or Igκ.[10,11]

In the present study, an approach to raising high-affinity, fully human mAb using a unique mouse radiation chimera system, the Trimera, is described. The Trimera system enables the adoptive transfer of human peripheral blood lymphocytes (PBL) into normal strains of mice[12,13] or rats[14] which were lethally irradiated and radioprotected with severe combined immunodeficient (SCID) mouse bone marrow. It was previously shown that human lymphoid cells engrafted in the Trimera could be induced to generate primary and secondary humoral responses as well as cellular responses against allogeneic lymphocytes and viral proteins.[12–16]

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Abbreviations: Ab, antibody; EBV, Epstein–Barr virus; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; mAb, monoclonal antibody; PBL, peripheral blood lymphocytes; PWM, pokeweed mitogen; SCID, severe combined immunodeficient.

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The utility of the Trimera system as a tool for generation of fully human mAb was investigated. We have used this system to develop human mAb to hepatitis B virus (HBV), a pathogen which presents a major public health problem worldwide.[17] A memory response to hepatitis B surface antigen (HBsAg) was elicited in Trimera mice engrafted with human lymphocytes from anti-HBV-positive donors.

Immunization of these mice with HBV vaccine resulted in marked amplification of the specific human antibody response to HBsAg. Stable hybridoma clones were derived from human B cells isolated from Trimera spleens, secreting high-affinity, fully human anti-HBsAg mAb. The development and characterization of these mAb in terms of their affinity and specificity are described and their potential therapeutic value is discussed.

## MATERIALS AND METHODS

*Mice*

BALB/c and SCID/NOD mice 6–10 weeks old were obtained from the Weizmann Institute Animal Breeding Center (Rehovot, Israel). All mice were fed sterile food and acid water containing cyprofloxacin (20 μg/ml) (Bayer, Leverkusen, Germany). Whenever necessary, mice were injected daily with 1 mg Fortum intraperitoneally (i.p.) for 5 days post bone marrow transplantation (Glaxo Operations UK, Greenford, UK).

*Peripheral blood lymphocytes*

Human PBL were obtained by leukopheresis after informed consent from donors positive for anti-HBsAg antibodies and negative for HBsAg, HBV-DNA, anti-hepatitis C virus (HCV) and anti-human immunodeficiency virus (HIV) antibodies. PBL were separated on a Ficoll–Hypaque gradient (UNI-SEP maxi; Eldan Tech., Jerusalem, Israel), washed twice, counted, and resuspended in phosphate-buffered saline (PBS).

*The Trimera system*

BALB/c mice were exposed to split dose total body irradiation (4 Gy followed 3 days later by 10–11 Gy), from a gamma beam 150-A $^{60}$Co source (produced by the Atomic Energy of Canada, Kanata, Ont., Canada) with focal skin distance (FSD) of 75 cm and a dose rate of 0·7 Gy/min. Following irradiation, each recipient mouse was immediately injected intravenously (i.v.) with $4–6 \times 10^6$ of SCID/NOD bone marrow cells,[18] and i.p. with $100 \times 10^6$ human PBL. On the same day of PBL transplantation, Trimera mice were immunized once i.p. with 2·5 μg/mouse hepatitis B vaccine (Engerix®-B; SB Biologicals Rixensart, Belgium). Non-immunized mice were injected with 0·15% Aluminium Hydroxide Gel (Merck, Danmstadt, Germany) in PBS.

*Cell and plasma collection from Trimera mice*

Animals were bled from the retro-orbital vein using heparin-coated glass capillaries. Plasma was kept for human-immunoglobulin determination. Animals were sacrificed by cervical dislocation, their spleens were removed, cut into pieces, and pressed through stainless steel sieves to make a cell suspension in PBS.

*Fluorescence-activated cell sorter (FACS) analysis of engrafted human cells*

Spleen cells were incubated for 30 min on ice with mouse anti-human CD45-fluorescein isothiocyanate (FITC) (pan leucocyte antigen, Caltag Laboratories, South San Francisco, CA). After washing, fluorescent analysis was performed on a FACScan analyser (Becton Dickinson, Mountain View, CA).

In vitro *activation of PBL*

PBL were separated from granulocytes and erythrocytes on a Ficoll–Hypaque gradient (UNI-SEP maxi; Eldan Tech., Jerusalem, Israel) and subsequently stimulated for 3–4 days with pokeweed mitogen (PWM) (Gibco BRL, Life Technologies Inc. Grand Island, NY) diluted 1:100 and with recombinant HBsAg (Bio-Hep-B,[19] kindly provided by Dr Naomi Moav, BioTechnology General, Rehovot, Israel) at 200 ng/ml in complete RPMI medium: RPMI-1640 medium supplemented with 10% (v/v) fetal calf serum (FCS), 10 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 1% (v/v) non-essential amino acids (Biological Industries, Beit Haemek, Israel) and $10^{-4}$ M-2-mercaptoethanol (2-ME; Sigma, St. Louis, MO).

*Cell fusion*

Cells were mixed with the human–mouse heteromyeloma HMMA2.11TG/0[20] at 3:1 ratio. Fusion was performed with 50% (w/v) PEG 1500 (Boehringer Mannheim GmbH, Mannheim, Germany) according to a standard procedure.[21] Fused cells were seeded at a concentration of 30 000 cells/well in 96-well U-bottom microtitre plates (Nunc, Roskilde, Denmark) in complete RPMI medium containing hypoxanthine, aminopterin and thymidine (HAT) supplement (1x) (Biological Industries, Beit Haemek, Israel). Cells were fed with fresh HAT-medium 1 week later. Two weeks after fusion, supernatants were harvested for enzyme-linked immunosorbent assay (ELISA) of human immunoglobulins and antigen-specific antibodies. Medium was replaced with fresh hypoxanthine, thymidine (HT)-containing medium. Hybridoma cultures secreting specific anti-HBsAg antibodies were cloned by limiting dilution at 0·5 cell/well in 96-well U-bottom microtitre plates.

*Determination of human immunoglobulin*

Trimera mice sera were tested for total human immunoglobulin and for antigen-specific antibodies. Total human immunoglobulin was quantified by sandwich ELISA using purified goat $F(ab)_2$ anti-human IgG+IgM+IgA (Zymed Laboratories, San Francisco, CA) as the capture agent (0·1 μg/well) and purified goat anti-human IgG+IgM+IgA or IgG peroxidase-conjugates (Zymed Laboratories) as the detection reagent. Immunoglobulin-calibrated human serum was used as the standard (Sigma, Rehovot, Israel). Concentration of HBsAg-specific human antibodies in mice sera was determined by ETI-AB-AUK-3 kit (Sorin Biomedica Diagnostics, Saluggia, Italy) or by quantitative HBsAb EIA kit (ZER, Jerusalem, Israel). Calibrators were standardized against World Health Organisation anti-hepatitis B immunoglobulin (1st International Reference Preparation, 1977).

Human immunoglobulins in hybridoma supernatants were determined by overnight incubation of supernatants on goat anti-human IgG+IgA+IgM (Zymed) coated plates

(0·1 μg/well), with goat anti-human IgG peroxidase-conjugate as the secondary reagent.

HBsAg-specific antibodies in hybridoma supernatants were determined as above using recombinant HBsAg-(Bio-Hep-B, BioTechnology General, Rehovot, Israel) coated plates (0·1 μg/well).

Absorbance at 450 nm was measured by an ELISA reader (Dynatech, Port Guernsey, Channel Islands, UK).

*Determination of human IgG subclasses*

Human IgG subclasses were determined by sandwich ELISA using purified goat $F(ab)_2$ anti-human IgG+IgM+IgA (Zymed Laboratories, San Francisco, CA) or HBsAg-coated plates (0·1 μg/well) for capture. Mouse anti-human IgG subclasses (Sigma) were used as second antibodies and purified goat anti-mouse IgG peroxidase-conjugate (Zymed Laboratories) as the detection reagent.

*Competitive inhibition assay*

Inhibition assay was performed in microtitre plates (F96 Maxisorp Nunc-Immuno Plate, Nunc, Denmark) coated with 0·05 μg/well HBsAg subtype ad (Chemicon International Inc., Temecula, CA). Purified anti-HBsAg antibodies were serially diluted from 800 to 1·5 ng/ml. Fifty microlitres of each dilution were added to the coated wells and subsequently, 50 μl (10 μg/ml) of HBsAg subtype ad (Chemicon International Inc.), recombinant HBsAg (Bio-Hep-B, BioTechnology General, Rehovot, Israel), recombinant hepatitis B-core antigen (HBcAg) (Chemicon International Inc.) or 50 μl of PBS alone. After 2 hr incubation at 37° and washings, 50 μl of goat anti-human IgG peroxidase-conjugate were added to each well. One hour after incubation at room temperature and washings, substrate solution 3,3′,5,5′-tetramethyl-benzidine dihydrochloride (TMB, Sigma) was added. Results were read using an ELISA reader, with a wavelength of 450 nm.

*Affinity constant measurements of anti HBsAg mAb*

Affinity measurements of purified human mAb were carried out using the BIAcore 2000 instrument (Pharmacia Biosensor), according to the manufacturer's instructions. Dissociation rates were determined using the software (BIAevaluation 2.1) provided by the manufacturers.

Determination of affinity constants ($K_d$) of the different anti-HBs mAb to HBsAg (ad) in solution were performed according to Friguet *et al.*[22] The antibody concentration used was deduced from an ELISA calibration performed on the same plate. $K_d$ was calculated from the relevant Scatchard plot.

*Immunohistostaining with anti HBsAg mAb*

Liver fragments from an HBV-infected patient or from a normal donor were fixed in 4% neutral buffered formaldehyde for 24 hr and then embedded in paraffin using routine procedures. Slices of 4 μm thickness were cut from paraffin blocks and mounted on polylysine-coated slides. After deparaffinization and peroxidase quenching, staining was performed using Protein A-purified human anti-HBsAg mAb followed by biotinylated goat anti-human IgG (H+L) using Histostain-SP® kit (Zymed, San Francisco, CA), according to the manufacturer's recommendation. Control slides were stained with biotinylated goat anti-human IgG only. Mouse anti-HBsAg antibody was obtained from Zymed Labs, Inc.

*Repertoire analysis of human immunoglobulin transcripts*

RNA was isolated from $10 \times 10^6$ hybridoma cells using RNAsol B (TEL-TEX, Inc. Friendswood, Tx). Complementary DNA was prepared from 10 μg of total RNA with reverse transcriptase (RT) and oligo dT (Promega, Madison, WI) according to standard procedures in 50 μl reaction volume. Polymerase chain reaction (PCR) was performed on 1 μl of the RT reaction mixture with three $V_H$ primers:

5′-GGGAATTCATGA(G)AA(C)A(C)A(T)ACTG(T)T-GG(T)A(T)G(C)CA(T)C(T)C(G)CTC(T)CTG-3′

5′-GGGAATTCATGGAGC(T)TTGGGCTGAG(C)CTG-GG(C)TTTC(T)T-3′

5′-GGGAATTCATGGACTGGACCTGGAGGA(G)TCC-(T)TCTG(T)C-3′

$V_\lambda$ primer:

5′-GGGAATTCATGA(G)CCTGG(C)A(T)CC(T)CCTC-TCC(T)TC(T)CTG(C)A(T)C(T)C-3′

or $V_\kappa$5′ leader primer:

5′-GGGAATTCATGGACATGA(G)A(G)G(A)A(G/T)-C(T)CCA(C/T)A(G/C)GC(T)G(T)CAG(C)CTT-3′

The 3′ primers correspond to human constant regions:

$V_H$5′-GCGAAGCTTTCATTTACCCA(G)GAGACAGG-GAGAG-3′

$V_\lambda$5′-GCGAAGCTTCTATGAACATTCTGTAGGGG-CCAC-3′

$V_\kappa$5′-GCGAAGCTTCTAACACTCTCCCCTGTTG-AAGCTC-3′.

The PCR fragments were cloned into pGEM-T vector (Promega) and sequenced using an ABI 377 sequencing machine. Sequences were analysed by comparison to Genbank and by alignment to Kabat sequences.[23]

*Statistical analysis*

Statistical analysis was performed using the Stat View II program (Abacus Concepts, Inc., Berkeley, CA) on a Macintosh Quadra 605 or Microsoft Excel 5.0 (Microsoft) on a 486 $DX_2$ PC compatible. Student's *t*-test, analysis of variance (ANOVA) correlation and regression analysis were utilized to calculate probability (*P*) and correlation coefficient (*r*) values. Results are presented as mean ± standard error.

## RESULTS

### Induction of specific human antibodies to HBsAg in Trimera

Human PBL from donors positive for anti-HBsAg antibodies were implanted i.p. into irradiated BALB/c mice which were radioprotected by transplantation of bone marrow from SCID mice. The resulting Trimera mice were then immunized with hepatitis B vaccine (Engerix B) to induce a secondary immune response. The production of specific human anti-HBsAg antibodies along with total human immunoglobulin secretion was measured in mice sera. Total human immunoglobulin and specific human antibodies to HBsAg started to be detectable 7 days following immunization with levels reaching a plateau after 2–3 weeks (data not shown).

Figure 1 shows the levels of total human immunoglobulin as well as the levels of specific human anti-HBsAg antibodies measured in mice sera 14 days after transplanting PBL from a single donor. The levels of human immunoglobulin secreted were similar in immunized and control non-immunized

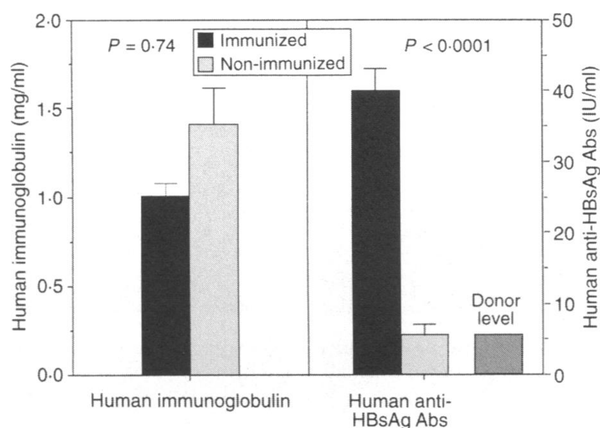


**Figure 1.** Levels of total human immunoglobulin and of human anti-HBsAg antibodies in Trimera mice sera. Trimera mice were immunized with 2·5 µg Engerix-B and were bled 14 days later. Levels of human immunoglobulin and of anti-HBsAg antibodies were measured in mice sera as described in the Materials and Methods. Trimera mice in the control group were transplanted with PBL and injected with 0·15% alum in PBS only.

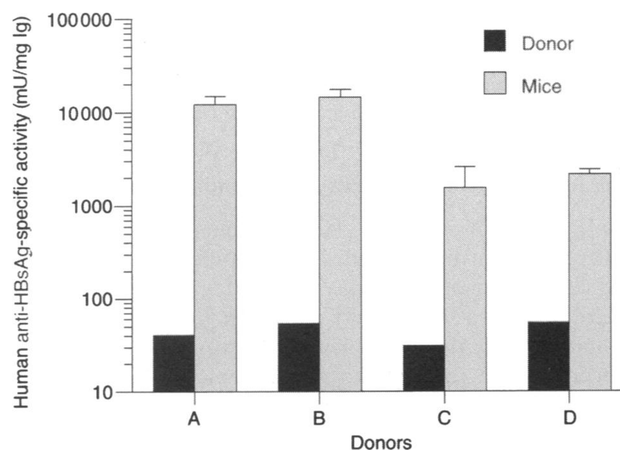


**Figure 2.** Human anti-HBsAg specific activity (anti-HBsAg immunoglobulin/total immunoglobulin) in Trimera mice sera. Trimera mice were transplanted with PBL from different donors (A–D) positive for anti-HBsAg antibodies. Mice were then immunized with Engerix-B and bled 14 days later. Levels of total human immunoglobulin and levels of anti-HBsAg antibodies were measured in mice and donors sera as described in the Materials and Methods. The specific anti-HBsAg antibody fraction was calculated as the levels of anti-HBsAg specific antibodies (in units of activity) per mg of human immunoglobulin.

Trimera ($P=0{\cdot}074$). In contrast, a strong specific immune response to HBsAg developed only in hepatitis B vaccine immunized mice ($P<0{\cdot}0001$), indicating memory immune response. We have also compared the levels of anti-HBsAg antibodies in donor and Trimera mice sera. The levels of specific antibodies in non-immunized Trimera mice were similar to those in the donor's serum, however, an average of eight-fold increase in anti-HBsAg immunoglobulin levels was found in sera of immunized Trimera mice as compared to that in the donor (Fig. 1). In several other experiments, using PBL from different donors, we have compared the anti-HBsAg-specific activity, i.e. the levels of anti-HBsAg-specific antibodies per mg of human immunoglobulin secreted, in mice and donors sera. The anti-HBsAg-specific activity in mice sera 14 days after transplantation ranged from 4 IU/mg to 80 IU/mg while that observed in the human donors sera ranged only from 0·04 IU/mg to 0·2 IU/mg indicating a $10^2$–$10^3$-fold increase in the specific antibody fraction (Fig. 2). This increase demonstrates a dramatic amplification of anti-HBsAg antibody production in response to the antigen in the Trimera mice.

### Generation of hybridoma clones

Two weeks after immunization, cells from spleens of the responding Trimera mice were harvested and analysed by FACS. Spleens having more than 10% human CD45-positive cells were used for fusion to human–mouse heteroymeloma cells to generate hybridoma clones. The clones were screened for their growth rate, total immunoglobulin secretion, and specific antibody production. Fusion frequencies in different experiments ranged from $0{\cdot}9\times10^{-5}$ to $5\times10^{-5}$. A high percentage (more than 70%) of the growing hybridoma clones secreted human immunoglobulin of which 0·1–4% produced specific human anti-HBsAg antibodies. The ability to isolate hybridomas from Trimera spleens was compared to that of the donors lymphocytes activated *in vitro* with PWM and HBsAg, in terms of immunoglobulin type and stability (Table 1). The majority of the hybridomas obtained from Trimera mice spleens were found to secrete IgG and were

**Table 1.** Secretion of IgG and IgM from hybridoma clones derived from Trimera spleen cells and from *in vitro*-activated PBL

| Source of hybridoma cells* | Anti-HBsAg secretors† | | Stability |
|---|---|---|---|
| | IgG | IgM | |
| *In vitro*-activated PBL | 23 (48%) | 25 (52%) | 1 stable for > 18 months<br>47 unstable |
| Trimera splenocytes | 8 (72%) | 3 (28%) | 7 stable for > 18 months<br>4 unstable |

*PBL were activated *in vitro* and then fused as described in the Materials and Methods. Cells from Trimera spleens were prepared and fused as described in the Materials and Methods.

†Hybridoma clones were tested for their ability to secrete anti HBsAg antibodies of IgG or IgM type as described in the Materials and Methods.

stable for more than 18 months (seven out of 2700 wells). In contrast, hybridomas derived by direct fusion of donor lymphocytes were mostly unstable, yielding only one clone (out of 5440 wells) that has been stable for more than 18 months.

### Characterization of human anti-HBsAg antibodies

Stable hybridoma clones secreting human IgG specific for HBsAg were further expanded. The antibodies secreted were purified by protein A affinity chromatography and characterized for isotype and affinity (Table 2). All were found to be of human IgG1 isotype. None of the mAb generated in the Trimera system reacted with goat-anti-mouse IgG (data not shown). High-affinity constants for HBsAg binding were determined by BIAcore and ELISA, all ranged between $10^{-9}$–$10^{-10}$ M (Table 2). Specific activity of the purified mAb was calculated and found to be in the range of 300–500 IU/mg IgG. Specificity of these mAb for the HBV surface antigen was tested both by a competitive inhibition assay and by immunohistostaining of human liver tissue infected with HBV. In the competitive inhibition assay we used two types of recombinant HBsAg and recombinant HBcAg as a non-relevant control (Fig. 3). Only the HBV surface antigens inhibited the binding of the mAb to the HBsAg-coated wells.

**Table 2.** Affinity constants of human anti-HBsAg mAb*

| Clone | Type | Affinity BIAcore $K_d$ (M) | Affinity ELISA $K_d$ (M) |
|---|---|---|---|
| 17.1.41 | IgG1 | $7{\cdot}6\times10^{-10}$ | $1{\cdot}34\times10^{-9}$ |
| 14.18.5 | IgG1 | $6{\cdot}1\times10^{-9}$ | $6{\cdot}1\times10^{-9}$ |
| 19.92.17 | IgG1 | $9{\cdot}2\times10^{-10}$ | $2{\cdot}91\times10^{-9}$ |
| 19.71.67 | IgG1 | $3{\cdot}2\times10^{-9}$ | $1{\cdot}75\times10^{-9}$ |
| 19.70.15 | IgG1 | $3{\cdot}2\times10^{-10}$ | $3{\cdot}01\times10^{-9}$ |
| 19.79.5 | IgG1 | $5{\cdot}0\times10^{-10}$ | $2{\cdot}62\times10^{-9}$ |

*Human anti-HbsAg mAb from different hybridoma clones were purified by protein A affinity chromatography. Antibodies were tested for IgG subtype and affinity as described in the Materials and Methods.

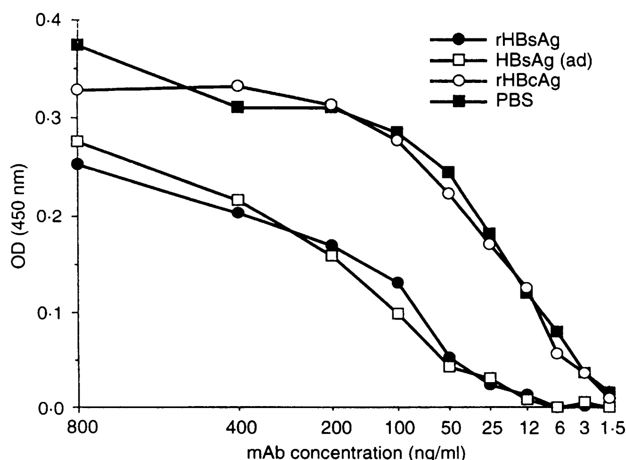


**Figure 3.** Representative competitive inhibition assay. The binding of serial dilutions of mAb 19.79.5 to HBsAg-coated plates was competed by addition of 0·5 μg of soluble HBsAg subtype ad (□), rHBsAg (●), rHBcAg (○), or PBS (■) as described in the Materials and Methods.

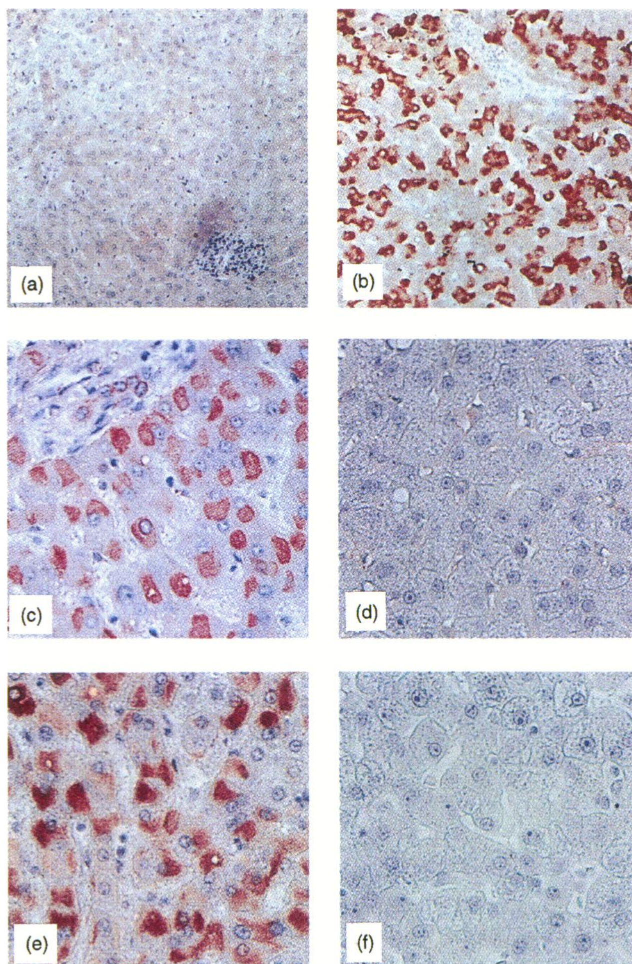


**Figure 4.** Binding of human anti-HBsAg mAb to human liver tissue infected with HBV as compared to normal liver. Human liver tissues either infected with HBV or virus-free were fixed in formaldehyde and stained with human mAb followed by biotinylated goat anti-human antibody and StrepAvidin peroxidase. (a) HBV-infected liver negative control, goat anti-human antibody and StrepAvidin peroxidase alone (×80); (b) HBV-infected liver positive control, commercial mouse anti-HBsAg mAb (×80); (c) HBV-infected liver stained with mAb 19.79.5 (×320); (d) normal liver stained with mAb 19.79.5 (×320); (e) HBV-infected liver stained with mAb 17.1.41 (×320); (f) normal liver stained with mAb 17.1.41 (×320).

Moreover, competition analysis by ELISA suggest that the antibodies do not compete for the same binding site, indicating that they recognize different epitopes (data not shown). In addition, these mAb bind only to the HBV-infected liver tissue and not to the normal control liver (Fig. 4). The staining intensity of infected hepatocytes with these mAb was high and comparable to that obtained with a commercial mouse mAb, indicating strong binding to the viral antigen.

The genes encoding the variable regions of these antibodies were isolated, fully sequenced and subgroups and complementarity-determining regions (CDR) were determined. All have fully human immunoglobulin gene sequences as determined by alignment to Genbank sequences and Kabat protein sequences.[23] Figure 5 demonstrates three representative mAb obtained from distinct experiments in which lymphocytes from three individual donors were used. The antibodies consist of

(a) $V_L$ amino acid sequences

| | FW1 | CDR1 | FW2 | CDR2 | FW3 |
|---|---|---|---|---|---|
| clone 17.1.41 | DIVMTQSPLSLSVTPGEPASISC | RSSQSLLHRSGNNYLD | WYLQKPGHSPQLLIY | VGSNRAS | GVPDRFSGSGSGTEYTLRISTVEAEDVGVYYC |
| clone 19.79.5 | SY*L**-*P*V**A**KT*R*** | GGNNIGTKNVH | **Q****QA*V*VV* | AD*D*P* | *I*E*****N**NTA**T**R**VG*EAD*** |
| clone 42.11 | **Q*****F***ASV*DRVT*T* | *A***INFYLN | **Q****KA*K**** | AA*TLQ* | ***S*********HF**T**SLQPD*FAT*** |

| | CDR3 | FW4 |
|---|---|---|
| clone 17.1.41 | MQALQTPRT | FGQGTKLEIKR |
| clone 19.79.5 | QVWDSVSYHVV | **G**T*TVLG |
| clone 42.11 | QHTYE**PYT | ******V**** |

(b) $V_H$ amino acid sequences

| | FW1 | CDR1 | FW2 | CDR2 | FW3 |
|---|---|---|---|---|---|
| clone 17.1.41 | QVQLVESGGGVVRPGRSLRLSCAASGFAFS | DYSIN | WVRQAPGKGLEWVA | IISYDGRITYYRDSVKG | RFTISRDDSKNTLYLQMNSLRTEDTAVYYCAR |
| clone 19.79.5 | ************Q**G*******P***V*R | S*GMH | ****T********S | L*WH**SNRF*A***** | *******N*************A****M*F*** |
| clone 42.11 | ***V*Q***E*KK**S*VKV**K***GP*N | N*A** | *******Q****MG | G*FPLFGTAKNAPRFQD | *V**IG*K*SS*V*MELRT**S*********S |

| | CDR3 | FW4 |
|---|---|---|
| clone 17.1.41 | QYYDFWSGSSVGRNYDGMDV | WGLGTTVTVSS |
| clone 19.79.5 | ERLIAAPAAFDL | **Q**L***** |
| clone 42.11 | G***-**EGDILH-*-*L** | **Q******** |

(c)

| Clone | Subgroups | | | |
|---|---|---|---|---|
| | $V_H$ | $J_H$ | $V_L$ | $J_L$ |
| 17.1.41 | $V_{H3}$ | $J_{H6}$ | $V_{\kappa 2}$ | $J_{\kappa 2}$ |
| 19.79.5 | $V_{H3}$ | $J_{H2}$ | $V_{\lambda 3}$ | $J_{\lambda 3}$ |
| 42.11 | $V_{H1}$ | $J_{H6}$ | $V_{\kappa 1}$ | $J_{\kappa 1}$ |

**Figure 5.** Sequence analysis of variable domains of human anti-HBsAg mAb. RNA from three different hybridoma clones was amplified by PCR, cloned and sequenced as described in the Materials and Methods. (a) $V_L$ amino acid sequences; (b) $V_H$ amino acid sequences; (c) V/J subgroups. Homology sequence to clone 17.1.41 is represented by *.

different $V_H$ genes and $J_H$ regions corresponding to various subgroups. The light chains consist of different $V_\kappa$ or $V_\lambda$ genes located in front of different $J_\kappa$ or $J_\lambda$ genes (Fig. 5c). These results demonstrate the ability to generate diverse antibodies from a normal human repertoire.

## DISCUSSION

In the present study we describe the generation of high-affinity, fully human mAb using the Trimera system. Lubin *et al.* described the engraftment of human lymphocytes in normal strains of mice and rats.[12–14] The engrafted lymphocytes colonized different mouse lymphatic organs very rapidly and were capable of mounting humoral responses to recall antigens such as tetanus toxoid and HBsAg as well as primary response to keyhole limpet haemocyanin in the host.[16] We have further established this system to obtain high-affinity fully human mAb to HBV which may have therapeutic potential.

The strong specific human immune response to HBsAg detected in the Trimera system could be attributed to overproduction of antibodies by mature human B cells that are maintained in the mouse environment. However, the fact that the specific antibody fraction in mice was 2–3 logs higher than that in the donor implies proliferation of memory B-cell clones induced by the antigen. This strong induction of memory suggests the presence of a functional immune system consisting of T helper cells, memory and mature B cells and antigen-presenting cells.

Human lymphocytes transplanted into SCID mice have been shown to produce immunoglobulins, respond to antigens, and survive for a long period of time.[24] However, whether these lymphocytes can actually repopulate and reconstitute lymphoid structure and organs has been subjected to some debate. In SCID mice the homing process from the site of transplantation, i.e. the peritoneum, to the lymphatic organs is very slow.[25] In addition, other drawbacks in using human–SCID mice have been reported including restricted B-cell repertoire and development of T-cell anergy.[26–28] Recently it was shown that better engraftment in SCID mice could be achieved after total body irradiation with 3 Gy followed by treatment with antiasialo GMl.[29] However, in our laboratory, the irradiated SCID mice mounted a weaker response to HBV as compared to our Trimera system (I. Lubin *et al.*, manuscript in preparation). It is possible that the previously functional environment of the normal mouse spleen supports propagation of human memory B cells leading to the formation of affinity mature antibodies. This supporting environment is probably lacking in the spleen of a SCID mouse that is not able to form germinal centres.[30] Further indication for this possibility is evidenced in the formation of primary follicular centres by human lymphocytes in the Trimera mouse spleen as reported by Burakova *et al.*[31]

Engrafted human B cells were concentrated in lymphatic organs forming, together with the engrafted human T cells, mixed lymphoid follicles. The presence of human B and T lymphocytes in close proximity to each other in the mouse lymphoid tissues may explain the ability of our Trimera mice to mount significant human antibody responses.

Human cells harvested from Trimera mice spleens were fused to generate hybridoma clones. We have isolated several specific hybridomas which are stable and secrete high-affinity human mAb of IgG class. These antibodies have different subtypes and subgroups of human origin, indicating diversity of the human immunoglobulin gene repertoire.

In the past, human mAb were derived from *in vitro*-activated human B cells fused to various fusion partners. The major hurdles in generation of human mAb centred around the source of pre-immunized B lymphocytes and the choice of fusion partner.[32] Human B cells were immortalized either by EBV transformation alone[33] or by combination of EBV and fusion to mouse myeloma cells.[3] EBV transformation of human B cells tends to produce unstable IgM lines with poor antigen affinity[34] while human–mouse hybridoma clones lead to preferential loss of human chromosomes and instability of the hybrids.[35] This was confirmed by our data showing that hybridomas generated by *in vitro*-activated B cells were mostly IgM and were less stable than those derived from Trimera spleens (Table 1). The role of the supporting environment of the Trimera mouse spleen on the stability of human hybridoma clones should be further investigated.

Hepatitis B infection can be prevented with polyclonal antibodies extracted from pooled hyperimmune human sera (HBIG). Individuals considered for such immunotherapy are those with continuous risk of hepatitis B infection (patients and staff of haemodialysis units), those who were exposed to a single contamination with hepatitis B-positive material (needle or cut injury), and neonates born to HBV-infected mothers.[36] In addition, passive immunotherapy could be useful to prevent recurrent infection in immunosuppressed patients who underwent liver transplantation.[37] High doses of HBIG were shown to neutralize the virus.[38] However, use of immunoglobulins collected from human plasma is limited in amount and present a safety issue. Human mAb to HBV generated by fusion of B cells derived from PBL of vaccinated patients have been described.[39–41] One such antibody was used clinically and showed neutralizing capability, however, neutralization of the virus was not complete and escape mutants emerged.[42] Thus, it seems that the use of a single mAb may be problematic therapeutically and that a mixture of several mAb might be more efficient.[43]

The activity of our antibodies is higher than that of commercially available polyclonal anti-HBsAg immunoglobulins, 300–500 IU/mg versus 0·5–1 IU/mg. The strong cytoplasmic staining of HBsAg in hepatocytes from infected human tissue by our mAb indicates binding to the surface protein of the virus. In addition, we have initiated experiments to assess the potential neutralizing activity of the human anti-HBsAg mAb generated in the Trimera system. These mAb are currently being tested for their ability to inhibit HBV infection of human liver tissue. In an HBV animal model developed in our laboratory, such infected liver fragments are engrafted in Trimera mice and appearance of HBV DNA is followed in mice sera. In this model, 80–85% of the mice become infected with HBV. A significant reduction in the rate of infection is seen in the presence of the mAb, with only 13–35% of the mice being infected with HBV (E. Ilan *et al.*, manuscript in preparation). Experiments are in progress to characterize further the function and neutralizing ability of these mAb.

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