

Llama-Derived Single-Domain Intrabodies Inhibit Secretion of Hepatitis B Virions in Mice

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Hepatitis B virus (HBV) infections cause 500,000 to 700,000 deaths per year as a consequence of chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Efficient and safe antivirals to treat chronically infected patients and consequently to prevent development of hepatocellular carcinoma are still awaited. We isolated five single-domain antibodies (VHHs) that recognize the most abundant envelope protein (S) of HBV. VHHs, when expressed and retained in the endoplasmic reticulum as intrabodies, reduced levels of secreted hepatitis B surface antigen (HBsAg) particles in a cellular HBV model. In a hydrodynamics-based HBV mouse model, these intrabodies caused a marked reduction in HBsAg concentrations and a 10- to >100-fold reduction in the concentration of HBV virions in plasma. Conclusion: VHHs potently inhibited secretion of HBV virions *in vivo*, showing that this approach might be useful in the treatment of HBV. To our knowledge, this is the first report of intrabody-mediated inhibition of viral secretion in mammals. (HEPATOLOGY 2009;49: 39-49.)

Hepatitis B virus (HBV) imposes an important burden to human health worldwide. Approximately 350 to 400 million persons are chronically infected. Among these, 15% to 40% will develop cirrhosis, liver failure, and/or hepatocellular carcinoma, resulting in 500,000 to 700,000 deaths each year.^{1,2} Potent and safe antivirals to successfully treat chronically infected HBV patients and to prevent development of hepatocellular carcinoma are still awaited. To date, only interferon-2 β , pegylated interferon-2 α , and four nucleos(t)ide analogues have been approved for the treatment of chronic HBV infections. However, interferons are only effective in a fraction (20%–40%) of patients and cause

numerous side effects. The licensed synthetic nucleos(t)ide analogues induce fewer side effects, but their therapeutic success is hampered by the appearance of drug-resistant mutants during long-term treatment. Discontinuation of treatment with nucleos(t)ide analogues generally leads to renewed viral replication that can be followed by acute exacerbation of liver disease.^{3–6}

Novel strategies to combat viral infections are being explored by several research groups. One approach is the ablation of viral messenger RNA expression by antisense oligonucleotides, peptide aptamers, ribozymes, and RNA interference–mediated gene silencing.³ Another approach consists of the intrabody-mediated inhibition of viral protein functions or interactions. Intrabodies are antibody fragments that are expressed and function inside the cell.^{7,8} Intrabodies can be targeted to a specific cell compartment such as the endoplasmic reticulum (ER), mitochondrion, nucleus, or cytoplasm. The most explored intrabody format is the single-chain variable fragment (scFv), which is derived from the variable antigen-binding domains of a conventional antibody. scFv intrabodies that interfere with viral replication have been developed against human immunodeficiency virus,⁹ hepatitis C virus,^{10,11} rotavirus,¹² herpesvirus,¹³ flavivirus,¹⁴ and HBV.^{15,16}

Single-domain antibodies (VHHs) are a new generation of recombinant antibody fragments. VHHs consist of the functional variable domain of heavy-chain-only antibodies of the family Camelidae, which are devoid of light chains.^{17–19} VHHs are the smallest intact antigen-

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; HBcAg, hepatitis B core antigen; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NC, negative control; PBS, phosphate-buffered saline; pHBsAg, plasma-purified HBsAg; scFv, single-chain variable fragment; VHH, single-domain antibody.

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binding domain fragments known. They have several interesting features. VHVs can recognize structures not recognized by conventional antibodies, such as enzymatic pockets and clefts. Compared with scFv, VHVs have a more simple structure and are smaller in size (11–15 kDa). They are also much more hydrophilic, which results in increased solubility, higher stability, and improved expression in heterologous systems. These qualities make them excellent candidates to be used as therapeutic intrabodies. Yet this potential application of VHH-based intrabodies has only been described twice *in vitro*.^{20,21}

We report the isolation of several single-domain intrabodies directed against the HBV S protein. This protein was chosen because it is the major envelope component of infectious viral particles and noninfectious hepatitis B surface antigen (HBsAg) particles.²² These VHVs, when expressed and retained in the ER, potently inhibited secretion of HBV virions in mice. To our knowledge, this is the first report of introbody-mediated inhibition of viral secretion in mammals, showing that this approach may be useful in the treatment of HBV and in addition to other viral diseases.

Materials and Methods

Construction of a VHH-Phage Display Library and Selection of Five S-Specific VHVs. A detailed description of this protocol can be consulted online at the HEPATOLOGY website (Supporting Data).

Purification of Monomeric VHVs. For production of soluble VHVs, the nonsuppressor *Escherichia coli* [E. coli] strain WK6 was used. Cells were grown at 37°C in 250 mL Terrific Broth-0.1% glucose-100 µg/mL ampicillin (Sigma) until an absorbance between 2 and 4 was obtained; 1 mM isopropyl-β-thio-galactoside and 5 mM MgSO₄ was then added. After 4 hours at 37°C, cells were pelleted and periplasmic proteins were extracted via osmotic shock. VHVs were purified via Ni²⁺-affinity chromatography (Talon Metal Affinity resin, BD Biosciences).

Purified VHH Enzyme-Linked Immunosorbent Assay. Maxisorp plates were coated with plasma-purified HBsAg (pHBsAg) (Biodesign, 1 µg/mL) and blocked with phosphate-buffered saline (PBS)/1% bovine serum albumin. After three washes with PBS/0.1% Tween 20, VHVs were added at the concentrations indicated. Afterward, mouse anti-myc was added (Roche), followed by anti-mouse horseradish peroxidase (HRP) conjugate (Sigma). HRP activity was determined with tetramethylbenzidine substrate (Sigma). After addition of 1 N H₂SO₄, the optical density was read at 450 nm.

Pseudobivalent VHH Enzyme-Linked Immunosorbent Assay. Pseudobivalent enzyme-linked immunosorbent assay (ELISA) is identical to the purified VHH ELISA, except that VHVs were incubated with a fixed concentration of mouse anti-myc prior to addition to the HBsAg-coated plates.

Multivalent VHH ELISA. Maxisorp plates were coated with 10 µg/mL purified VHVs and blocked with PBS/1% bovine serum albumin. After three washes, HBsAg was added at the concentrations indicated. Next, 1 µg/mL biotinylated rabbit anti-HBsAg (Biodesign) was added, followed by streptavidin-HRP conjugate (Sigma).

Cloning of VHVs in the pShooter Vector, pCMV/ER/myc. VHH sequences were amplified using one of the forward primers (FW-ER) and the reverse primer (RV-ER) (Supporting Table 1). PCR fragments were digested with NotI and BssHII and ligated into the pCMV/ER/myc plasmid (pShooter, Invitrogen). TOP10 *E. coli* cells (Invitrogen) were transformed with this ligation product.

Cells and Transfections. HepG2 cells were grown in cRPMI medium (RPMI medium, 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, 2 mM L-glutamine) at 37°C in a humidified 5% CO₂ atmosphere. Twenty-four hours before transfection, cells were transferred to cRPMI medium with 0.1 mM dithiothreitol and 10 mM dextrose. Transfection of 4 × 10⁶ cells with 20 µg plasmid was performed via electroporation at 250 V and 975 µFd in 300 µL cRPMI (without fetal bovine serum) with 0.1 mM dithiothreitol and 10 mM dextrose. The electroporated cells were resuspended in 9 mL of cRPMI and transferred to 3 wells of a 6-well plate. For immunostaining purposes, transfected cells were seeded on coverslips. Three to four hours after transfection, supernatant was replaced completely by fresh medium. Supernatant (750 µL) was collected and replaced by fresh medium 1, 2, and 3 days after electroporation. HepG2 cells were harvested via EDTA/trypsin treatment. After two washes with PBS, the cells were lysed during five freeze/thaw cycles in 200 µL PBS-1× Complete Protease Inhibitor Cocktail (Boehringer Mannheim). After centrifugation, the protein concentration of the cleared cell lysate was determined (Protein Assay Dye Reagent, Bio-Rad).

HBV Hydrodynamic Mouse Model. The use of this model was approved by the animal ethical committee of the Faculty of Medicine, Ghent University, Belgium (ECD 05/39). Scid (CB^ySmn.CB17-Prkdc^{scid}) mice were bred and maintained under specific pathogen-free conditions in the mouse facility of the Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University. Male mice (9–12 weeks old) were anesthetized

with ketamine (Ketalar, Pfizer) and xylazine (Rompun, Bayer). Twenty micrograms of plasmid DNA in 1.5 mL PBS was injected within 5 seconds into the tail vein. Plasma samples were isolated to quantify HBsAg, hepatitis B e antigen (HBeAg), and HBV DNA. To isolate the liver, mice were euthanized via cervical dislocation. The liver was divided into two parts: one part was preserved in 4% formalin for immunohistochemical analysis; the other was sliced into three pieces that were stored at -80°C . To prepare liver lysates, one part was thawed and cut into 10 similar-sized pieces. Two pieces were added to 200 μL 10 mM Tris-HCl (pH7.6)-1 \times Complete Protease Inhibitor Cocktail and lysed by 10 freeze/thaw cycles. After centrifugation, the protein concentration of the cleared lysate was determined.

Analysis of HBV and VHH Expression in Transfected Cells and Mice. VHH expression in cell and liver lysates was investigated via western blotting. Different HBV parameters (HBsAg, HBeAg, and HBV DNA) were determined in cell culture supernatant, plasma, and lysates. Finally, immunohistochemical study on HepG2 cells and liver slices was performed. A detailed description of all these procedures can be obtained at the HEPATOLOGY website (Supporting Data).

Results

Isolation of Llama VHH Fragments via Phage Display. To isolate VHHs that bind the viral membrane proteins, two llamas were immunized with noninfectious pHBsAg particles. Peripheral blood cells and cervical lymph node cells from each of these llamas were used to construct a VHH-phage display library that contained $\approx 10^7$ individual transformants. These transformants harbor the phagemid pAX004 with a cloned nucleic acid VHH sequence, in frame with both a six-histidine (his) tag and a myc tag at the C-terminus. Separated by an amber stopcodon, this sequence is followed by the phage coat geneIII. The VHH repertoire of the library was expressed on phages after infection of the bacteria with the helperphage M13K07.

Selection of S-Specific VHHs via Biopanning. During infection, three structural viral membrane proteins (S, M, and L) are translated into the ER of infected hepatocytes. These proteins share 226 C-terminal amino acids, called the S-domain. In addition, the M protein has an N-terminal preS2-domain of 55 amino acids. The L protein also contains this preS2-domain, preceded by the preS1-domain that consists of a supplementary 108 or 119 amino acids. Two types of viral particles are secreted by the infected hepatocyte: the infectious virions or Dane particles and the noninfectious lipoprotein particles (HB-

sAg). S is the most abundant membrane protein in all these particles.²² Therefore, we selected the S-domain/protein as a target molecule for the intrabody-mediated inhibition of the replication cycle.

To select VHHs that specifically bind the S-domain and not the preS2 or preS1 domain, biopanning was performed with recombinant HBsAg, which consists solely of S protein. After two rounds of biopanning, 90 bacterial colonies were randomly picked and induced for the secretion of phages displaying several VHH molecules on their surface (up to five) or for the secretion of soluble VHH molecules. These were screened for target specificity via phage ELISA and VHH screening ELISA, respectively. Whereas 83% of the phages bound to recombinant HBsAg, only 45% of the soluble VHH molecules scored positive.

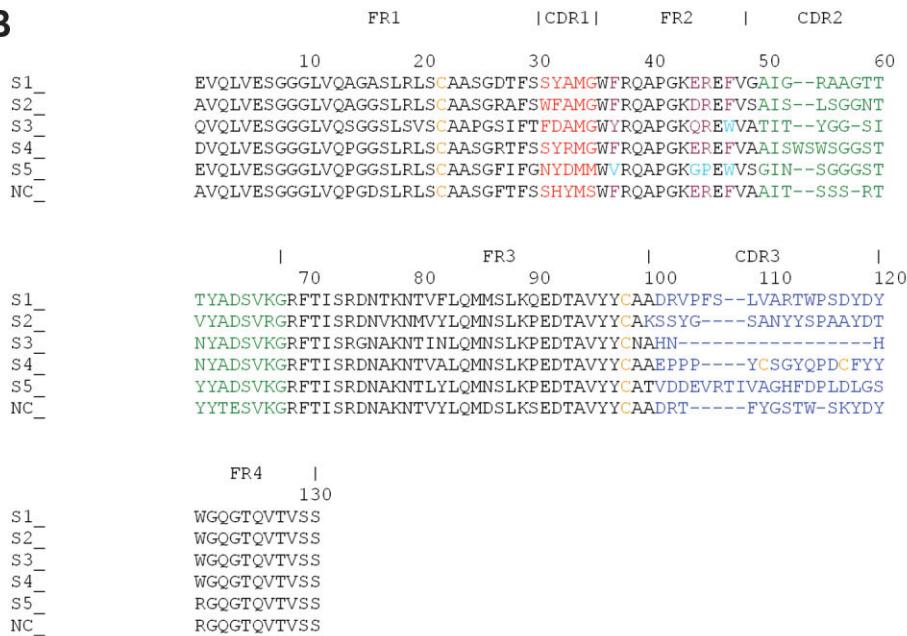
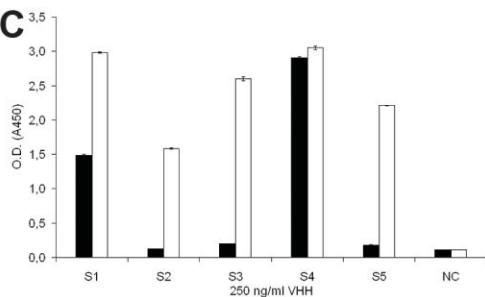
Colony polymerase chain reaction confirmed the presence of VHH coding phagemids in the bacteria. These were all analyzed at DNA levels via *HinfI* fingerprinting. Thirty-five VHH coding genes with a different restriction pattern were sequenced, aligned, and subdivided in 20 families. Based on their sequence and on their antigen-binding capacity, five S-specific VHHs were selected (S1-S5). VHHs S1, S3, and S4 bound HBsAg in the ELISA using soluble VHH proteins, while the other two S-specific VHHs (S2 and S5) bound only the antigen in the phage format (Fig. 1A). Each of the selected amino acid VHH sequences had interesting features (Fig. 1B). Although they successfully passed the biopanning selection, some VHHs scored negative in both the phage ELISA and the VHH screening ELISA. One of these VHHs was chosen as a negative control (NC) for further experiments.

Antigen-Binding Properties of the Selected VHHs. After production and purification of monomeric molecules of the selected VHHs, their antigen-binding characteristics were first studied via purified VHH ELISA (Fig. 1A). The previous screening data were confirmed for VHHs S1, S2, S3, and S4. Unexpectedly, VHH S5 did not bind the antigen at all in the VHH screening ELISA, but yielded a weak positive result in the purified VHH ELISA. The very weak or even negative result of some of the VHHs (S2, S3, and S5) in the VHH screening ELISA and the purified VHH ELISA indicated a lower affinity of these VHHs. The binding was much better in the phage ELISA because a phage displays on average 2 to 5 VHH molecules, which conveys an increased avidity and an improvement of the antigen-binding properties.

To enhance the functional affinity of the purified VHHs, pseudobivalent complexes were constructed. This was achieved by incubating VHHs with mouse anti-myc at stoichiometric correct concentrations before adding them to the immobilized antigen. Calculation of the cor-

A

	phage ELISA (*)	VHH screening ELISA (*)	Purified VHH ELISA (**) (2 µg/ml VHH) (n=3)
S1	1.841	0.365	0.511 ± 0.046
S2	1.888	0.096	0.156 ± 0.026
S3	1.848	0.344	0.208 ± 0.029
S4	1.882	0.591	1.636 ± 0.055
S5	1.966	0.091	0.224 ± 0.031
NC	0.036	0.065	0.150 ± 0.009
-	0.019	0.055	0.154 ± 0.013

B**C**

rect stoichiometry was based on the difference in molecular weight between a VHH (16 kDa) and a conventional antibody anti-*myc* (165 kDa) (ratio 1:10) and on the need to occupy the two binding sites of one anti-*myc* molecule with each one VHH molecule (ratio 2:1), yielding a final ratio of ≈1:5 for the proportion of VHH/anti-*myc* molecules in micrograms per milliliter. A much better antigen binding was observed with these pseudobivalent constructs. Whereas incubation of VHHs S1 and S4 had only limited effects on the already significant binding of these VHHs to HBsAg, addition of anti-*myc* to S2, S3, and S5 drastically increased the binding capacity to HBsAg of these VHHs (Fig. 1C). The binding of NC remained unaltered.

Fig. 1. Binding characteristics and amino acid sequence of the five S-specific VHHs (S1-S5) and the non-S-specific VHH NC. (A) Binding of the VHHs to HBsAg in a phage ELISA, a VHH screening ELISA, and a purified VHH ELISA. The optical density was read at a wavelength of 405 nm (*) or 450 nm (**). (B) Alignment of the amino acid sequences. The framework regions (FR) and complementary determining regions (CDR) are indicated. Residues in light blue are typically human, while residues in purple are characteristic for camelids. Residues in orange represent cysteines that might form disulphide bridges. Each of the amino acid VHH sequences had interesting features. The most striking ones were the extreme short CDR3 with only three amino acids of S3, the typical human residues in the FR2 of S5, and the four prolines and two cysteines in the CDR3 of S4. The latter might give rise to a disulphide bridge in the CDR3. (C) Antigen binding of pseudobivalent VHH/anti-*myc* constructs at approximately stoichiometric correct concentrations of 1 µg/mL mouse anti-*myc* and 250 ng/mL VHH (n = 2). Black bars: no preincubation of the VHH with anti-*myc*. White bars: preincubation of the VHH with anti-*myc*.

Construction of Eukaryotic Vectors that Express VHH in the ER. To express the VHHs in eukaryotic cells, their coding sequence together with the adjoining his tag was cloned into the pCMV/*myc*/ER plasmid, in frame with an ER-targeting signal (a mouse V_H signal peptide) and the SEKDEL ER retention signal. The vector contains a *myc* tag before the SEKDEL for detection of the expressed protein by an anti-*myc* antibody. Sequencing of the constructs revealed the absence of the his tag coding sequence in VHH S1.

Effect of S-Specific VHHs on HBsAg Secretion in a Cellular Model. To study the effect of VHHs on HBsAg secretion, cotransfections of the hepatoma cell line HepG2 were performed. A total amount of 20 µg DNA

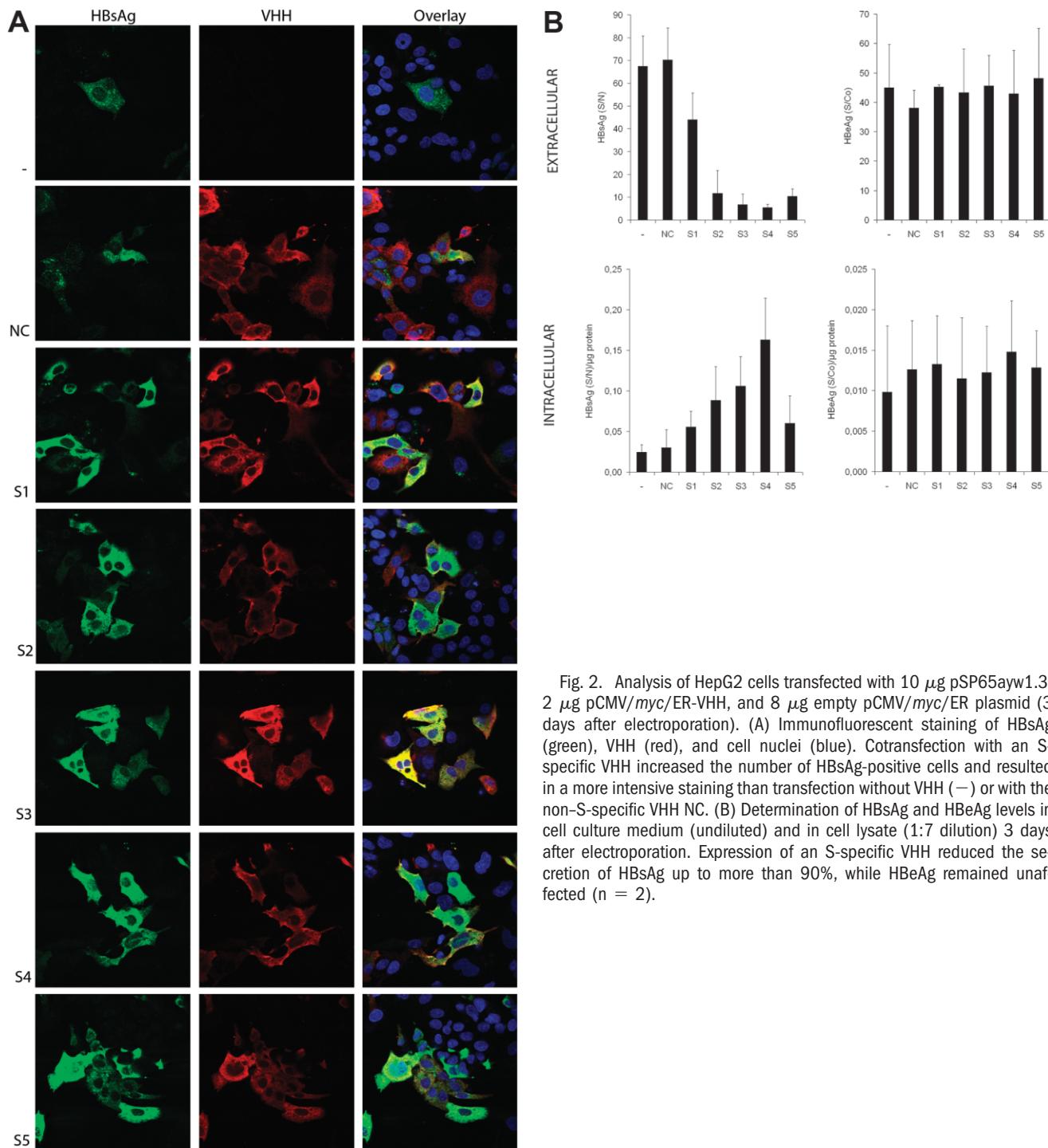


Fig. 2. Analysis of HepG2 cells transfected with 10 μ g pSP65ayw1.3, 2 μ g pCMV/myc/ER-VHH, and 8 μ g empty pCMV/myc/ER plasmid (3 days after electroporation). (A) Immunofluorescent staining of HBsAg (green), VHH (red), and cell nuclei (blue). Cotransfection with an S-specific VHH increased the number of HBsAg-positive cells and resulted in a more intensive staining than transfection without VHH (—) or with the non-S-specific VHH NC. (B) Determination of HBsAg and HBeAg levels in cell culture medium (undiluted) and in cell lysate (1:7 dilution) 3 days after electroporation. Expression of an S-specific VHH reduced the secretion of HBsAg up to more than 90%, while HBeAg remained unaffected ($n = 2$).

was introduced: 10 μ g HBV-expressing plasmid pSP65ayw1.3 (a kind gift from F. Chisari, The Scripps Research Institute), 2 μ g pCMV/myc/ER-VHH, and 8 μ g empty pCMV/myc/ER plasmid.

Confocal laser scanning microscopy of stained cells 3 days after electroporation revealed colocalization of HBsAg and VHH in the ER. Interestingly, when S-specific VHH DNA was cotransfected, more HBsAg-positive cells and a more intensive HBsAg staining were observed (Fig. 2A). In con-

trast, transfection without VHH DNA or with the non-S-specific VHH NC resulted in less HBsAg-positive cells and a less intensive staining. Although these observations were not quantitative, they suggest intracellular accumulation of HBsAg in association with S-specific VHH expression. VHH expression was also demonstrated via western blot analysis on cell lysates (data not shown).

Quantification of secreted HBsAg in the cell culture medium showed a decline in the level of secreted HBsAg

as early as 1 day after transfection, and this effect persisted on day 2 and 3 (Fig. 2B). The HBsAg level dropped more than 80% in VHHs S2 and S5, while for S3 and S4 the level dropped more than 90%. VHH S1 induced only a 35% reduction in secreted HBsAg. The level of HBeAg, another secreted HBV protein, was not affected (Fig. 2B). This demonstrated the specificity of the VHHs for HBsAg and suggested that VHHs do not affect protein secretion as a whole.

Accumulation of HBsAg inside the cell was confirmed by measuring HBsAg levels in cell lysates. To normalize these data, total protein content was determined. Transfection with pSP65ayw1.3 in the absence of VHH DNA or with the non-S-specific VHH NC resulted in a low amount of intracellular HBsAg, while higher HBsAg amounts per microgram total protein were measured when an S-specific VHH was expressed (Fig. 2B). The highest values were measured in VHH S4, but the increase was also considerable in S2 and S3. The intracellular amount of HBeAg per microgram total protein was comparable for the NC and the S-specific VHHs (Fig. 2B). This suggested again that VHH expression or VHH function has no inhibitory effect on protein secretion in general.

Taken together, these *in vitro* data demonstrate the capacity of the S-specific VHHs to suppress HBsAg particle secretion and increase HBsAg accumulation/retention inside the cell.

Anti-HBV Potential of VHHs In Vivo. The hydrodynamics-based transfection method is a quick, easy, and useful tool to create a mouse model for HBV infection.²³ We have applied this method to Scid mice to investigate the effect of S-specific VHHs on the replication of HBV. First, we performed a series of experiments with the same amounts of transfecting DNA as in the *in vitro* experiments (10 µg pSP65ayw1.3, 2 µg pCMV/myc/ER-VHH and 8 µg empty pCMV/myc/ER). Blood samples were collected at day 1, 4, 7, and 11 to quantify HBsAg, HBeAg, and HBV DNA. After animals were killed at day 1, 4, or 7, livers were isolated for immunohistochemical analysis.

No signs of VHH expression-induced liver damage was observed in any of the liver sections. Active caspase-3 staining showed absence of hepatocyte apoptosis, while smooth muscle actin staining revealed the absence of Kuppfer cell activation (data not shown). VHH immunostaining of liver sections showed VHH expression at day 1 after injection, but the number of VHH-positive cells already started to decrease on day 4 (Fig. 3A). On day 7 after injection, VHH-positive cells could no longer be observed (data not shown). Hepatitis B core antigen (HBcAg) was clearly visible, whereas HBsAg was much more difficult to detect (Fig. 3A), possibly because HBsAg

particles tend to be secreted and not retained inside the cell. However, more HBsAg-positive cells were observed on day 4, when an S-specific VHH was expressed. This suggested increased levels of intracellular HBsAg, indicating that the S-specific VHHs were capable of retaining S molecules inside the hepatocytes.

VHH expression was also visualized on western blot analysis in liver lysates, except for S1, which could not be detected due to the lack of the his tag (Supporting Fig. 1).

Levels of HBsAg and HBeAg were determined in plasma samples and liver lysates. In plasma a clear reduction in HBsAg level was measured up to 4 days post-injection compared to mice injected with HBV plasmid alone (Fig. 4A.1). This reduction was most prominent in VHHs S3 and S4. The reduction was specific, because injection of the non-S-specific VHH plasmid NC did not result in a reduced amount of secreted HBsAg. When measured in a 1:7 dilution, some of the HBsAg values were still at the plateau level (>80 S/N), especially for the injection without VHM DNA or with the non-S-specific VHH plasmid NC. Therefore, some of these samples were further diluted until values in the linear area were reached. Values exceeding an S/N of 1,000 could be detected (data not shown). Therefore, we assume that the difference in HBsAg levels between the S-specific VHHs and the controls are more prominent than suggested. In none of the experimental groups HBeAg levels were affected (Supporting Fig. 2). The decline in extracellular HBsAg with an S-specific VHH was reflected by an increased amount of intracellular HBsAg in the liver lysates at day 4, suggesting that HBsAg was retained in the hepatocytes (Fig. 4B.1). At day 7, only S3-transfected animals still maintained increased intracellular HBsAg levels.

More importantly, the concentration of HBV DNA in plasma, which corresponds to the number of secreted virions, was reduced up to 10-fold with S3 and S5 at day 7 after injection (Fig. 5A). VHHs S1 and S4 induced a less pronounced reduction in HBV DNA. For VHH S2, no significant reduction was observed at any time point. VHH NC exhibited no effect on HBV DNA levels, demonstrating the specificity of the S-directed VHHs.

To investigate a dose-response of VHH plasmid on the secretion of HBsAg and virions, 10 µg instead of 2 µg VHH plasmid was injected. VHHs S3 and S5 were chosen for this experiment because they yielded the strongest reduction in HBV DNA levels with 2 µg VHH plasmid.

Again, immunohistochemical analysis of liver sections revealed no signs of expression-induced liver damage. VHH, HBcAg, and HBsAg staining yielded similar results compared with the 2-µg injections, although a prolonged detection of VHH-positive cells and S-positive cells was observed up to day 7 (Fig. 3B). Once again,

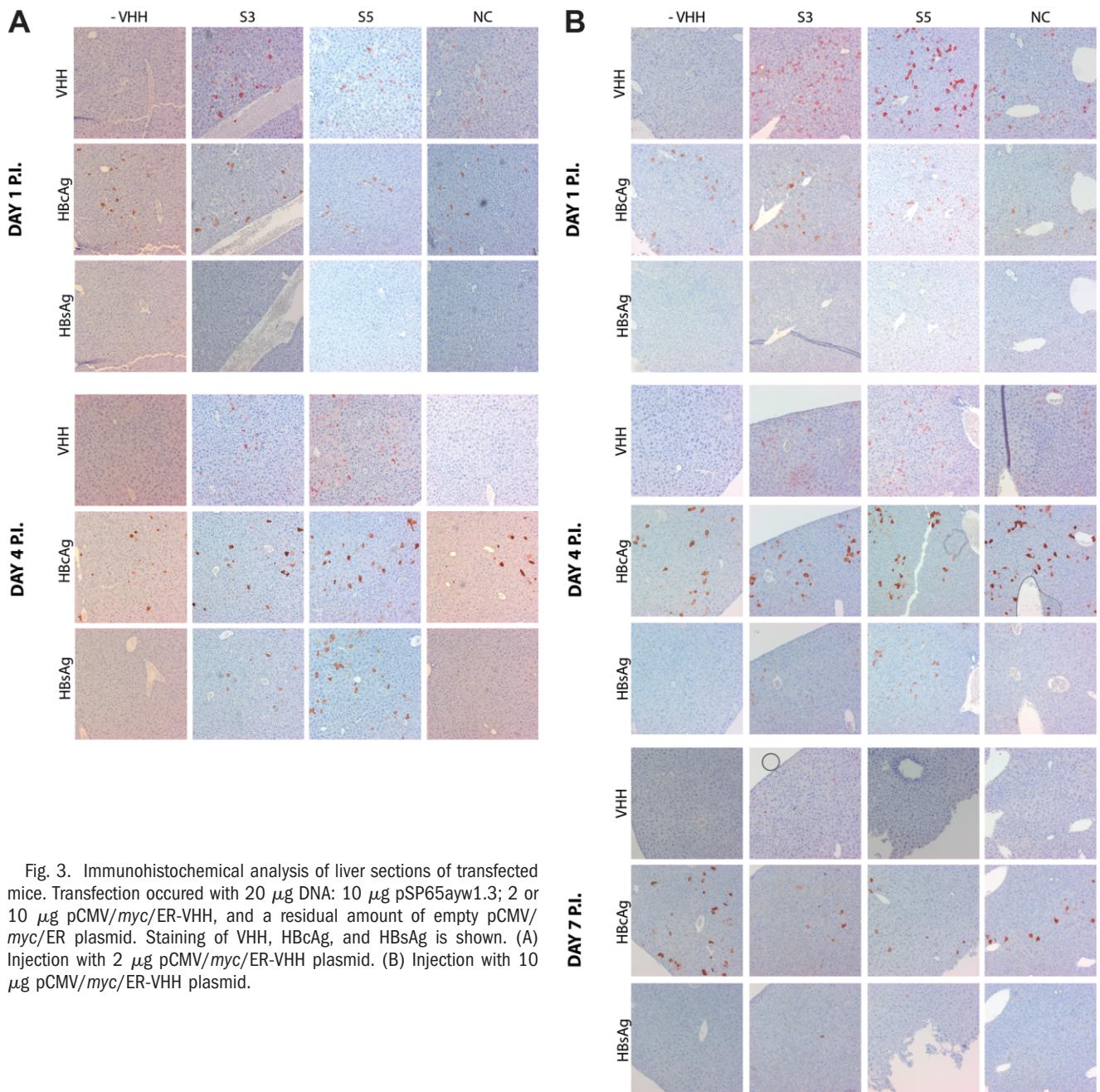


Fig. 3. Immunohistochemical analysis of liver sections of transfected mice. Transfection occurred with 20 μ g DNA: 10 μ g pSP65ayw1.3; 2 or 10 μ g pCMV/myc/ER-VHH, and a residual amount of empty pCMV/myc/ER plasmid. Staining of VHH, HBcAg, and HBsAg is shown. (A) Injection with 2 μ g pCMV/myc/ER-VHH plasmid. (B) Injection with 10 μ g pCMV/myc/ER-VHH plasmid.

VHH expression was confirmed via western blotting of liver lysates (Supporting Fig. 1).

With 10 μ g VHH DNA, the suppression in HBsAg secretion in the plasma lasted up to day 11 after transfection, especially for S5 (Fig. 4A.2). Again the levels of secreted HBeAg were not affected (Supporting Fig. 2). The intracellular HBsAg amount was increased for both S3 and S5 at day 4 after injection. At day 7, the values were already comparable with those found in controls (Fig. 4B.2).

Additionally, a much stronger inhibition of the secretion of viral particles was observed. HBV DNA levels in the

plasma of mice injected with S5 plasmid were reduced at least 100-fold at day 11 after injection (Fig. 5B), and this is likely an underestimation, since the plasma samples did not reach the detection limit of 3.16×10^4 copies/mL.

Antigen-Binding Capacity of Multivalent VHH Complexes. As mentioned above, expression of VHH S5 induced by the injection of 10 μ g VHH plasmid resulted in a severe suppression in virion secretion. This was quite surprising, because this particular VHH could not bind HBsAg in a monomeric format (Fig. 1A). Construction of pseudobivalent complexes improved antigen binding (Fig. 1C), probably as the result of an increased functional affinity.

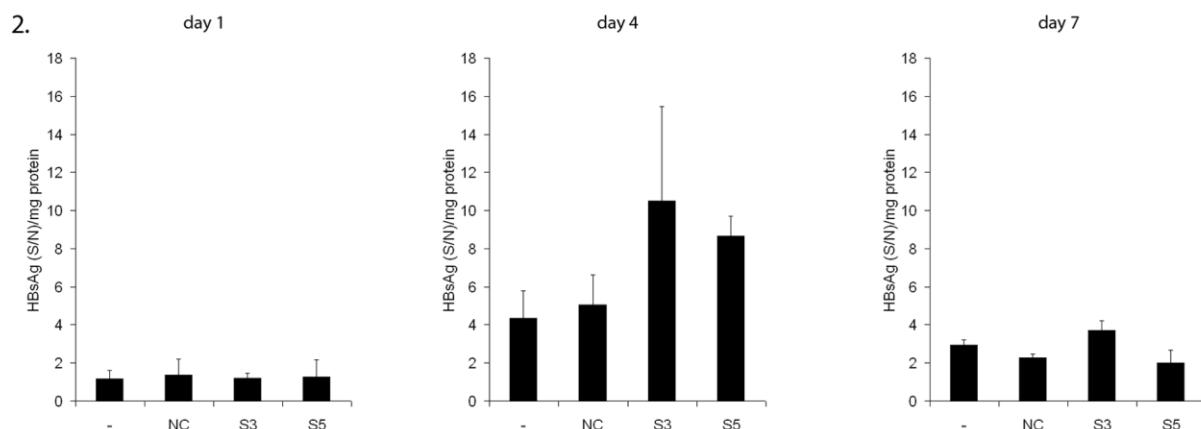
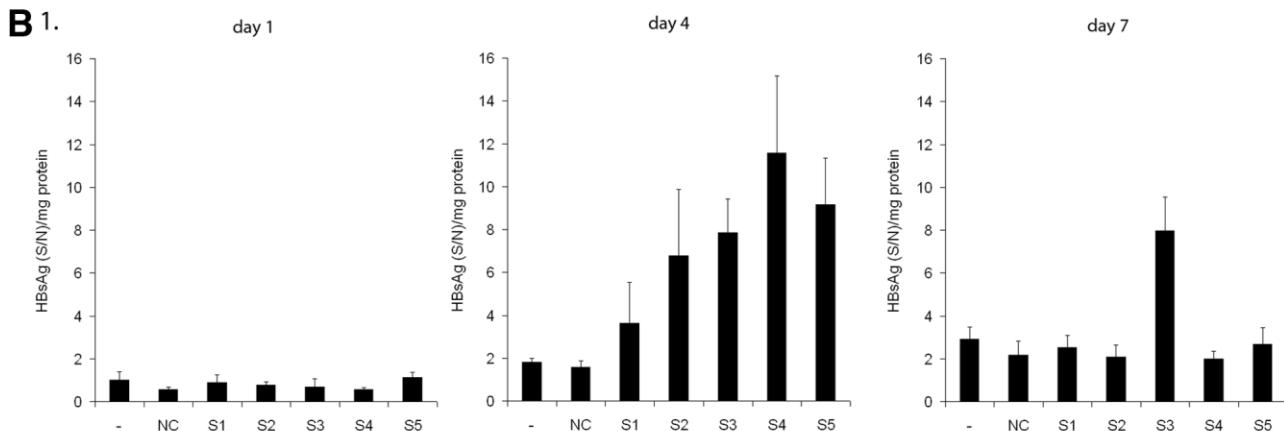
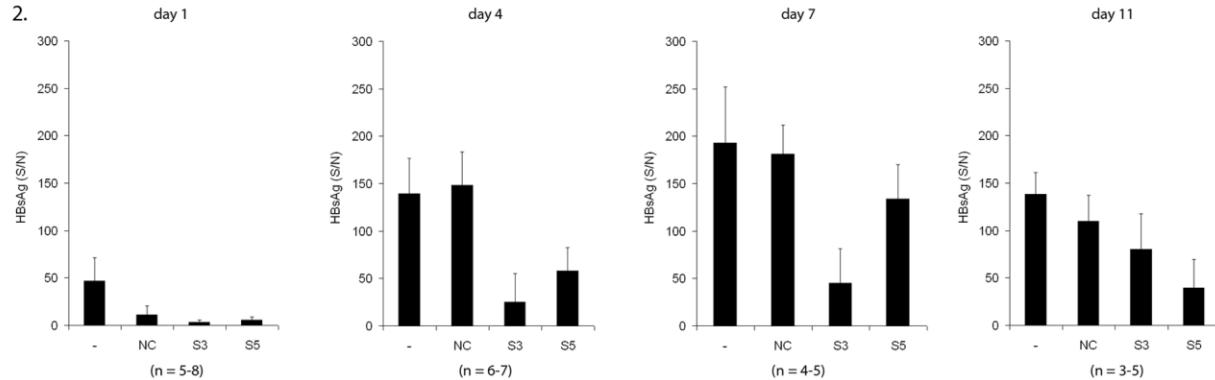
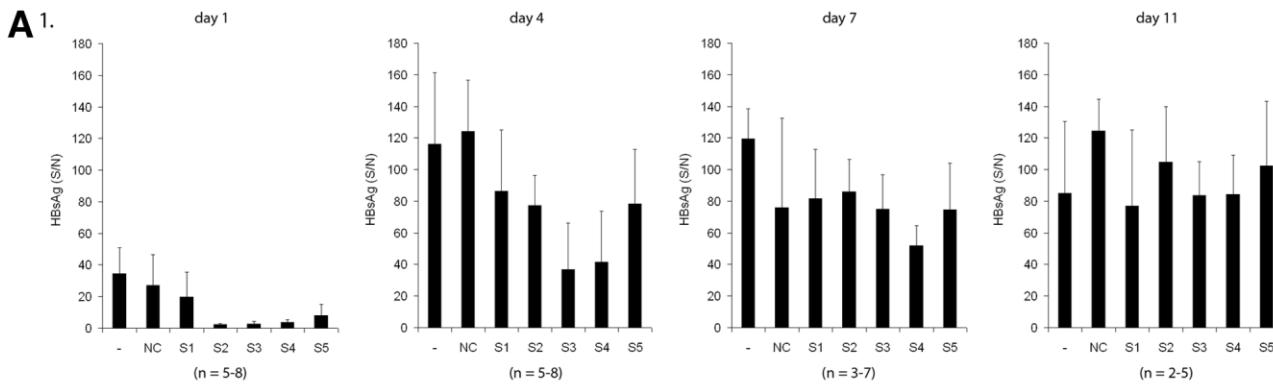


Fig. 4. HBsAg levels in (A) plasma and (B) liver lysates of transfected mice. Mice were injected with (1) 10 μ g pSP65ayw1.3 plasmid and 2 μ g pCMV/myc/ER-VHH plasmid or (2) 10 μ g pCMV/myc/ER-VHH plasmid. The minus sign (-) represents a control injection without pCMV/myc/ER-VHH plasmid. The samples were diluted 1:7 in fetal bovine serum. In plasma, a reduced level of secreted HBsAg was observed, whereas an increased amount was detected in liver lysates.

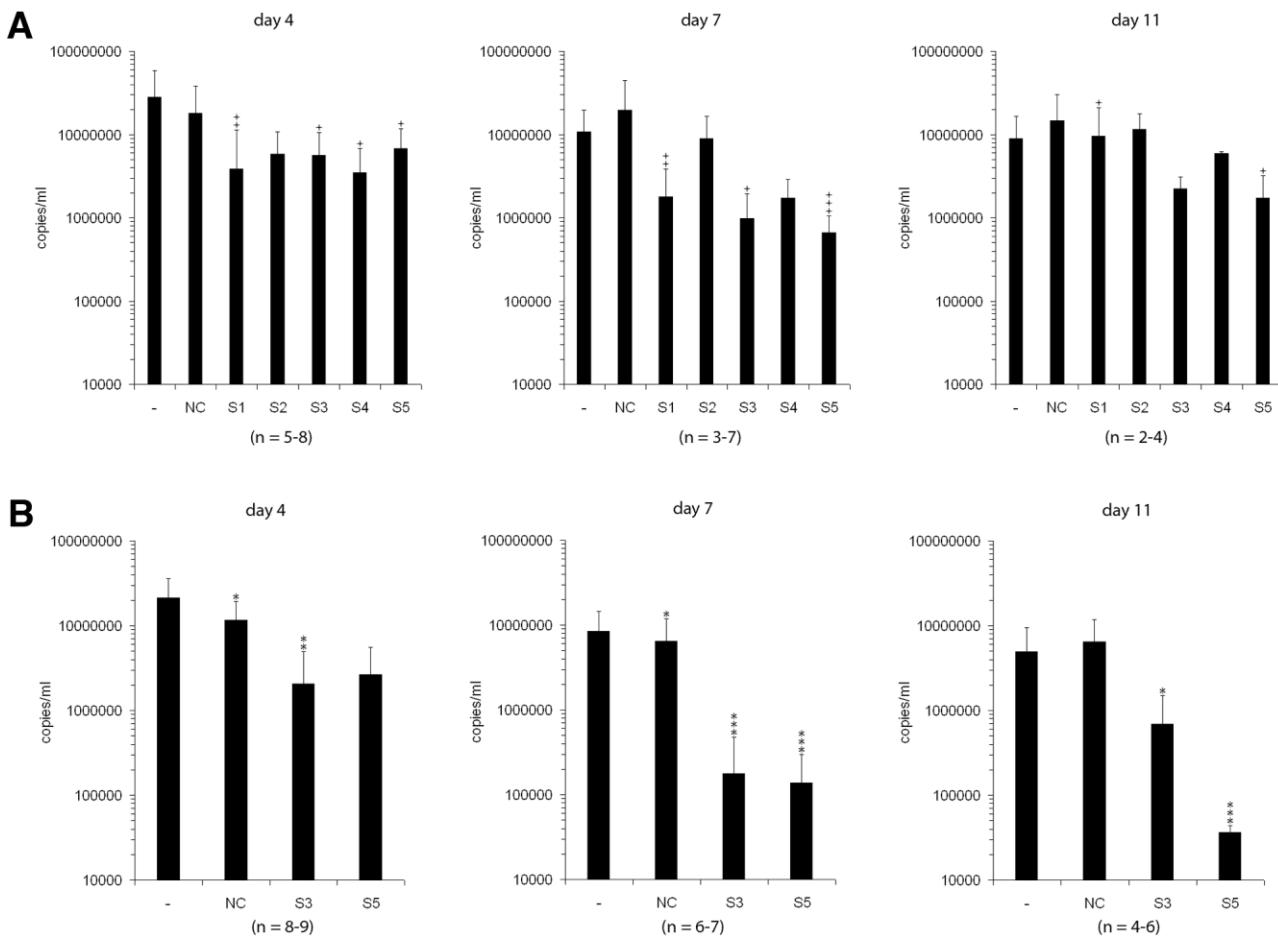


Fig. 5. Analysis of the secreted level of infectious particles in the plasma of transfected mice. Mice were injected with (A) 10 μ g pSP65ayw1.3 plasmid and 2 μ g pCMV/myc/ER-VHH plasmid or (B) 10 μ g pCMV/myc/ER-VHH plasmid. The level of HBV DNA in plasma was measured, which corresponds to the number of secreted virions. With 10 μ g pCMV/myc/ER-S5 plasmid, the level of secreted virions was reduced at least 100-fold. *One mouse under detection limit of 3.16×10^4 copies/mL. **One mouse under detection limit of 3.16×10^5 copies/mL.

We hypothesized that the numerous VHH molecules retained in the ER membrane of hepatocytes form a two-dimensional or even three-dimensional VHH array that is capable of binding the S-molecules that enter the ER with high avidity. This phenomenon may inhibit further transport and ultimately secretion of HBsAg particles and virions. To test this hypothesis, we investigated whether a multivalent complex of VHH molecules could bind the antigen in an efficient way, because of an extreme increase in avidity. Therefore, we studied the binding of HBsAg particles to VHHs coated on a Maxisorp plate, thus mimicking a VHH array. All S-specific VHHs, except S2, could bind the antigen efficiently (Fig. 6). VHH S5 was even able to bind HBsAg at concentrations as low as 3.5 ng/mL. These binding data resemble the *in vivo* data quite well, showing that S5 was the best inhibitor of virion secretion, while S2 was the least effective VHH (Fig. 5).

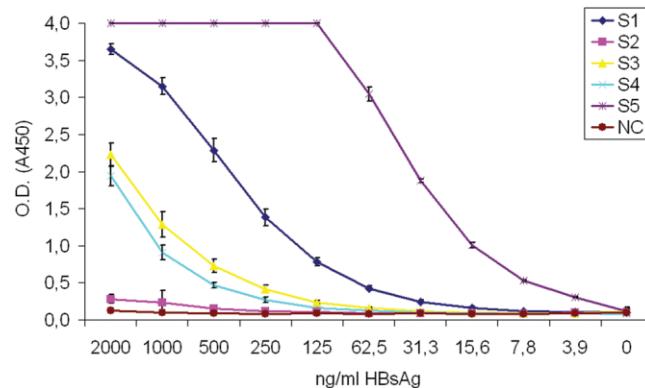


Fig. 6. Multivalent VHH ELISA to investigate the HBsAg binding of VHHs, coated on a plate at a concentration of 10 μ g/mL ($n = 3$). All VHHs except S2 bound well. VHH S5 could even bind HBsAg down to 3.5 ng/mL.

Discussion

In this study, the capacity of S-specific VHH intrabodies to interfere with the replication of HBV was conclusively demonstrated *in vitro* as well as *in vivo*. VHHs were chosen for this study over other formats of intrabodies such as Fab and scFv because of their superior properties, including their more simple structure and their more hydrophylic nature.

Inhibition of HBV replication by intrabodies has only been reported twice in *in vitro* studies with scFv. zu Pulitz et al.¹⁶ reported an S-specific scFv that resulted in an 85% reduction in HBsAg particle secretion. An HBcAg-specific scFv has also been developed, causing an efficient shutdown in viral DNA replication.¹⁵ In contrast to scFv, VHHs have rarely been explored as a strategy for viral inhibition.

In this study, a VHH-phage display library derived from immunized llamas was selected against HBsAg to obtain S-specific VHH molecules. First, binding properties of the isolated VHHs (S1-S5) were characterized via ELISA, and differences in affinity were presumed. Biacore analysis confirmed these ELISA-based observations (Supporting Table 2). For VHH S5, a kiss-and-run mechanism could even be assumed because of a high on-rate as well as a high off-rate. Despite these differences in affinity, we continued to work with all five VHHs, because the expressed VHHs inside the cell will be massively targeted to the ER. There they will most likely not act as monovalent molecules but as multivalent complexes with high avidities. Moreover, it has been previously reported that even low-affinity intrabodies may be efficient in inhibiting viral infections.¹³

In vitro and *in vivo* experiments revealed a decrease in secreted viral and nonviral particles, both in the supernatant of transfected HepG2 cells and in the plasma of injected mice. Confocal microscopy of HepG2 cells as well as immunohistochemistry of liver sections suggested an accumulation of HBsAg particles inside the cell when an S-specific VHH was expressed. The precise mechanism of action of the VHH that causes the decrease in viral and nonviral secretion remains unclear. At this point we do not know whether the inhibitory action of the S-specific VHHs takes place at the stage of the formation of complete particles or at a later stage. In the first case, it is possible that the interaction between VHH-retained S-molecules in the ER and the mature capsid in the cytoplasm is disrupted or that the interaction between individual S-molecules in the ER is blocked, both resulting in an arrest of particle formation. In the latter case, we hypothesize that complete particles are already formed inside the secretory pathway, but that further transport is inhibited by VHH retention in the secretory pathway.

Because the intracellular HBsAg particle level was not that much increased as expected from the massive reduction in extracellular level, it is unlikely that the inhibition is only due to the latter mechanism. Interactions of the VHHs with individual S molecules according to the first hypothesis may indeed occur, and because these individual S-molecules cannot be detected by the HBsAg (V2) AxSYM test, a lower than expected level of intracellular HBsAg level is observed.

Although the VHH molecules are massively targeted to the ER and will most likely act there as a multivalent layer, an even more severe retention of viral and nonviral particles might be obtained by using bivalent VHH intrabody constructs.

In this study, the VHHs were tested *in vivo* in an acute model by injecting both the VHH and HBV plasmid at the same time. Experiments will be performed to study the effect of the VHHs in a more chronic HBV model by first establishing an HBV infection, followed by the introduction of VHH expression. To study the contribution of the immune system in the clearance of HBV in a chronic infection, immunocompetent mice can be used.²⁴ Indeed, it is possible that strong HBV suppression caused by VHHs may restore the down-regulated immune system and lead to an immune response that is able to clear the virus. To achieve this, strong and long-lasting VHH expression is necessary. The currently used pShooter vector contains the strong but short-lived CMV promoter.²⁵ Liver-specific and long-living promoters such as the human α -antitrypsin promoter with its enhancers or the apolipoprotein A-I promoter may be more appropriate for applications that require more persistent VHH expression.²⁶

Overall, the data presented here provide proof of principle for the use of intrabodies to inhibit viral secretion *in vivo* and demonstrate the potential of VHHs in the treatment of HBV. In addition, these VHHs may also be a powerful tool to study viral entry, envelope production, and budding of HBV,²⁷ processes that are still poorly understood.^{28,29}

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