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In vivo activity of a mixture of two human monoclonal antibodies (anti-HBs) in a chronic hepatitis B virus carrier chimpanzee

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A 35-year-old female hepatitis B virus carrier chimpanzee was infused with one dose of a mixture of human monoclonal antibodies 9H9 and 4-7B (antibodies against hepatitis B virus surface antigen; HBsAg). Blood samples were taken before and up to 3 weeks after infusion. HBsAg and antibodies against HBsAg (anti-HBs) were quantified by radioimmunoassay and enzyme immunoassay. Free anti-HBs was never detected. Thirty min after the start of the infusion the HBsAg level was minimal with maximum loading of the chimpanzee HBsAg with human immunoglobulin. HBsAg complexes could be dissociated by acid treatment. The HBsAg level was completely restored on day 7. Similar results were obtained for the preS1-containing particles that may represent the infectious viral particles in the chimpanzee serum. A mouse monoclonal anti-HBs (HBs.OT40) was found to compete with 9H9 in artificial immune complexes with the pre-treatment HBsAg from the chimpanzee. Used as a conjugate, HBs.OT40 yielded a maximum decrease in the signal in the 30 min sample compared to non-competing anti-HBs conjugates. This indicates binding of HBsAg with 9H9 in the circulation of the chimpanzee. Immune-complexed 4-7B could not be detected by its corresponding 4-7B peptide conjugate, probably due to its low concentration in the complexes. It is concluded that human monoclonal anti-HBs can effectively reduce the level of HBsAg in serum from this chronic carrier. Monoclonals 9H9 and 4-7B may complement each other due to their different mechanisms of inactivation, probably with higher efficiency than that monitored by our HBsAg screening assays.

Introduction

Hepatitis B virus immunoglobulin (HBIg) has been used for more than a decade to prevent hepatitis B virus (HBV) infection in newborns of hepatitis B virus surface antigen (HBsAg)-positive mothers and in adults after needle stick accidents. More recently HBIg has been introduced as a prophylactic against recurrent hepatitis B in liver transplant recipients (Samuel *et al.*, 1993). Restricted availability of HBIg, partially due to the discovery of new blood-borne viruses such as human T-lymphotropic virus types 1 and 2, human immunodeficiency virus types 1 and 2, and hepatitis C virus has stimulated the introduction and application of (human) monoclonal antibodies (Ehrlich *et al.*, 1992).

Recently, HBsAg escape mutants were observed in vaccinated children (HBIg and HBV vaccine) (Oon *et al.*, 1996) and in liver transplant recipients treated with monoclonal as well as polyclonal anti-HBs (Carman *et al.*, 1996). An alternative treatment of liver transplant recipients with nucleotide analogues, which looked very promising at first, is hampered at present by the emergence of virus resistance caused by changes in the polymerase gene (Bartholomew *et al.*, 1997).

It has been suggested that a combination of carefully selected monoclonal antibodies could prevent the appearance of escape mutants. Recently, we studied the inhibitory activity

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of a mixture of two monoclonal antibodies *in vitro* (Heijtink *et al.*, 1995). One of these antibodies, 9H9, is directed against a conformational epitope located in the 'a' region, the other, 4-7B, is directed against a linear epitope at the C-terminal end of the S protein (W. P. Paulij and others, unpublished data). In HBV-positive serum from liver transplant recipients the 9H9 monoclonal antibody was effective against wild-type HBV whereas the 4-7B monoclonal antibodies could also 'neutralize' mutant HBV, including the Arg-145 variant (Busstra *et al.*, 1995).

Prior to administration of 9H9 and 4-7B antibodies to chronically infected hepatitis B patients we studied the effect of these monoclonal antibodies on the serum level of HBsAg (S protein) and HBV (represented as preS1 protein) in a chronic hepatitis B virus carrier chimpanzee.

Methods

■ **Anti-HBs treatment of the chimpanzee.** A 35-year-old female chimpanzee weighing 52 kg was HBsAg-positive, anti-HBs-negative, HBeAg-negative, anti-HBe-positive and HBV DNA-negative by solution hybridization. HBsAg was of the mixed ad/ay type with major reading of HBsAg/adw_2. Several treatments, such as administration of anti-HBs, interferon or a preS2-enriched HBsAg vaccine, were given to break the carrier state, unfortunately without success. The last treatment ended 5 years before the start of the present study.

The animal was housed at the Laboratory for Experimental Medicine and Surgery in Primates (LEMSIP) of the New York Medical Center, New York, USA. The study was approved by the LEMSIP Institutional Animal Care and Use Committee.

In January 1995 this chimpanzee was intravenously inoculated while under anaesthesia (ketamine hydrochloride, 10–15 mg/kg) with a 1:1 mixture on the basis of weight of human monoclonal anti-HBs 9H9 ($3{\cdot}64 \times 10^5$ IU) and 4-7B ($2{\cdot}20 \times 10^3$ IU) (Biotest Pharma) in 50 ml PBS with 1% human albumin. Pre-treatment samples were taken at both 2 months and 12 min prior to the start of therapy. Other samples were taken at 10, 20 and 30 min, 1 h, 6 h, 12 h and 1, 2, 4, 7, 14 and 21 days after the start of the infusion. The infusion was completed in 33 min. Serum samples were stored at −20 °C until use.

■ **HBsAg and anti-HBs assays.** HBsAg and anti-HBs levels were evaluated by commercial radioimmunoassays (RIAs) (Ausria II, Ausab IMx, Abbott Laboratories) and in experimental enzyme immunoassays (EIAs) according to standard procedures. Experimental EIAs were introduced for detection of preS1-containing particles and for detection of 9H9 and 4-7B antibodies specifically.

Assay protocols. In EIA plates solid phase antibodies (anti-S, anti-preS1) were used for capturing either HBsAg (S protein) or HBV (preS1 protein) from serum. The presence of captured S protein or preS1 protein was analysed or quantified by anti-S or anti-preS2 (in case of preS1-containing particles) conjugates. Human immunoglobulin (Ig) or mouse Ig present in immune complexes with S protein or preS1 protein was detected by anti-human Ig and anti-mouse Ig conjugates. These immune complexes were captured by solid phase anti-S or anti-preS1.

For quantification of artificial immune complexes we used the 'inhibition in solution assay'. In this assay antigen and antibody were preincubated for 2 h at room temperature and the residual detectable amount of antigen (S protein or preS1 protein) was measured using the standard antigen detection assay (Heijtink *et al.*, 1995).

To detect the preference for similar epitopes by two monoclonal antibodies from different species of origin, captured HBsAg (on solid phase anti-S) was subsequently loaded with the two antibodies. Binding preferences were visualized using species-specific anti-Ig conjugates.

Test components. In the EIAs solid phase antibodies were mouse monoclonal HBs.OT40 (anti-S) (Organon Teknika), a combination of three mouse monoclonals (anti-S), HBs.OT20, HBs.OT13 and HBs.OT16 (abbreviated as HBs.OT3M) (Organon Teknika), or sheep anti-preS1 (Organon Teknika). Mouse monoclonals HBs.OT40 (anti-S), HBs.OT42 (anti-S) (Organon Teknika) or OTB-anti-preS2 (Organon Teknika) were used as conjugate (biotin/avidin–HRP labelling, Amersham). HRP-labelled goat anti-human Ig (Sigma), sheep anti-human Ig (Amersham) and rabbit anti-mouse Ig (Dako) were used for detection of human Ig and mouse Ig in complexes. After peptide mapping the corresponding 4-7B peptide (Organon Teknika) was used as solid phase 'antigen' or as conjugate (HRP).

HBsAg and preS1-Ag were assayed in sequential serum samples from the chimpanzee. However, to ensure optimum assay systems and reference material in *in vitro* experiments, we used HBsAg from the hepatoma cell line HepG2 2.2.15 (ayw_3), human HBsAg (Mat.O[adw_2]; see Heijtink *et al.*, 1995) and HBsAg/ayw_2 (P2; Reference HBsAg, Paris, 1977) as well.

Polyclonal anti-HBs (Hepatect) was obtained from Biotest. Stock solutions of 9H9 and 4-7B contained 14550 IU/ml and 88 IU/ml, respectively. Since the dilution curves for 9H9 and 4-7B were different we expressed the concentration of 9H9 and 4-7B preferentially as a dilution factor for stock rather than in IU/l.

■ **Immune complex dissociation.** Immune complexes were degraded by the acid dissociating method of Nishanian *et al.* (1990). In short, 50 µl 0·5 M HCl was added to a 100 µl sample and incubated for 15 min at 37 °C. Subsequently, 50 µl 0·5 M NaOH was added and incubation was continued for another 5 min at room temperature. To stabilize the pH after NaOH treatment samples were diluted in 1 M Tris–HCl (pH 7·6) which did not influence the pH reduction achieved with 0·5 M HCl (to pH 1·9).

Results

HBsAg and anti-HBs in chimpanzee serum during treatment and follow-up

HBsAg was quantified in consecutive serum samples by means of Ausria II (Abbott Laboratories) and by an experimental EIA (Fig. 1). HBsAg was standardized with the Paul Ehrlich Institute standard for HBsAg. According to these two assays the pre-treatment sample contained 36 µg/ml (RIA) or 49 µg/ml (EIA) HBsAg.

Anti-HBs (Ausab IMx, Abbott Laboratories) could not be detected before (−12 min), during (10, 20 or 30 min) or after (1 h) infusion of monoclonal 9H9/4-7B. However, our experimental HBsAg EIA with solid phase mouse monoclonal HBs.OT40 (anti-S) could detect an increase in human Ig on the surface of the chimpanzee HBsAg in the first 24 h after infusion of monoclonal anti-HBs.

HBsAg binding in radioimmunoassay inhibited by 9H9 and 4-7B

Previously we had established the 50% inhibitory concentrations (IC_{50}) of 9H9 (110 IU/l) and 4-7B (12 IU/l) for HBsAg (Mat.O, 6 ng/ml) in human serum in an 'inhibition in solution

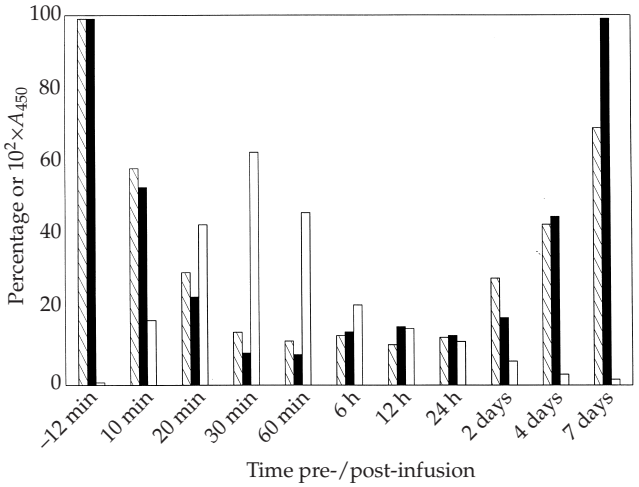

Fig. 1. Quantification of HBsAg from a chronically infected chimpanzee after infusion with a 1 : 1 (w/w) mixture of human monoclonal 9H9 and 4-7B by radioimmunoassay (hatched bars) (Ausria II, Abbott Laboratories) and an experimental HBsAg EIA (solid bars) using solid phase mouse monoclonal HBs.OT40 and conjugated mouse monoclonal HBs.OT42 (Organon Teknika). Infusion lasted 33 min. Immune-complexed human Ig was monitored simultaneously with the HBsAg EIA (unfilled bars) using goat anti-human Ig as conjugate and presented as absorbance at 450 nm.

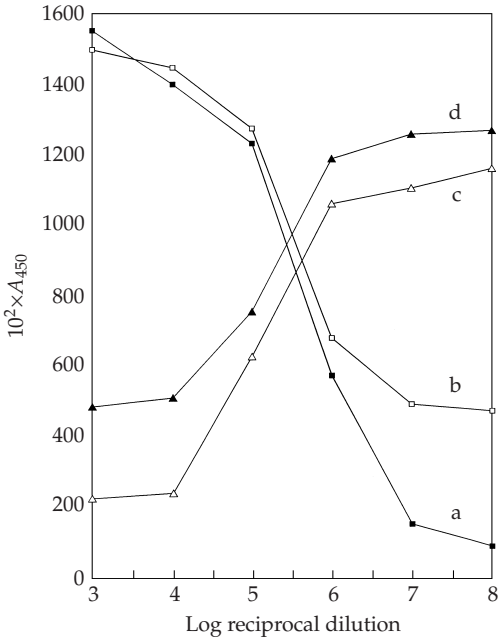

Fig. 2. Solid phase anti-preS1 was subsequently loaded with HBsAg/ayw$_2$ (P2), anti-S (9H9 and HBs.OT40) and conjugated anti-mouse Ig or anti-human Ig. HBsAg was applied to solid phase anti-preS1 in a fixed concentration (dilution 1/100); the second and third incubation steps were performed with titrated amounts of HBs.OT40 and a fixed amount ($1/10^5$) of 9H9 (curves a, d), respectively, and similarly with a titrated amount of 9H9 and a fixed amount of HBs.OT40 (curves b, c). Monitoring was by anti-mouse Ig (curves a, c) and anti-human Ig (curves b, d). Results are given in absorbance at 450 nm.

assay' (Heijtink *et al.*, 1995). In this assay, HBsAg is pre-incubated with anti-HBs and the residual detectable amount of HBsAg is measured by means of a commercial HBsAg assay (Ausria II, Abbott Laboratories). The 50% reduction rate is estimated from a titration curve. In a similar experiment with the chimpanzee HBsAg (pre-treatment sample) the same result was obtained for 9H9 (IC_{50} 110 IU/l 9H9 with 6 ng/ml chimpanzee HBsAg). Somewhat lower amounts of 4-7B (4 IU/l) were needed for chimpanzee HBsAg compared to human HBsAg (12 IU/l). In this experiment a maximum inhibition rate of 94% and 100% was obtained for 9H9 and 4-7B, respectively.

Binding of human HBsAg in EIA inhibited by 9H9 and 4-7B

In order to assess 9H9 and 4-7B in immune complexes we changed from the radioimmunoassay to an (experimental) enzyme immunoassay. In our experimental HBsAg EIA we started with mouse monoclonal HBs.OT40 (anti-S) as solid phase and mouse monoclonal HBs.OT42 (anti-S) as the conjugate. Both antibodies recognize conformational epitopes.

Unexpectedly, in experiments with our reference HBsAg (Mat.O) not only did the IC_{50} of 9H9 and 4-7B determined by EIA and RIA differ but the IC_{50} of 9H9 also decreased 500-fold relative to the IC_{50} of 4-7B. Replacing the solid phase HBs.OT40 (anti-S) in our EIA by a combination of three different anti-S-specific mouse monoclonals (HBs.OT20/HBs.OT13/HBs.OT16; abbreviated to HBs.OT3M) reduced the relative difference in IC_{50} between 9H9 (1/20480) and 4-7B (1/2048) for HBsAg–Mat.O binding by a factor of 10 (see Fig. 5*a*). Since the change in relative difference in IC_{50} was attributed predominantly to 9H9, these experiments suggest that HBs.OT40 and 9H9 may compete for the same epitope region on HBsAg. Due to the lower sensitivity of our experimental EIAs compared to the commercial RIA, larger amounts of HBsAg (RIA, 6 ng/ml; EIA, 70 ng/ml) and antibodies had to be used.

Mouse HBs.OT40 (anti-S) and human 9H9 react with similar epitopes on human and chimpanzee HBsAg

To confirm the affinity of mouse HBs.OT40 (anti-S) and human 9H9 for the same epitope region on HBsAg, experiments were performed with HBsAg/ayw$_2$ (P2, HBsAg panel of reference sera, Paris, 1977) on solid phase anti-preS1. Anti-preS1 was introduced to avoid competition between the anti-HBs antibodies in the samples and the anti-HBs in the solid phase. P2 was chosen because of its favourable preS1/S ratio and low level of background human Ig reactivity.

Solid phase sheep anti-preS1 was loaded with a fixed amount of HBsAg/ayw$_2$, which resulted in an absorbance of 0·600 with HBs.OT.42 (anti-S) as conjugate. After the incubation with HBsAg/ayw$_2$ this HBsAg was subsequently loaded with (human) 9H9 and mouse HBs.OT40 (anti-S), or vice versa, in separate incubation steps. Finally, the presence of human 9H9 or mouse HBs.OT40 (anti-S) was analysed with anti-human Ig or anti-mouse Ig conjugates, respectively. Fig.

Table 1. Human monoclonal 9H9 and mouse monoclonal anti-S (HBs.OT40) may use similar epitopes on chimpanzee HBsAg fixed to solid phase anti-preS1

In successive incubation steps chimpanzee HBsAg was bound to solid phase anti-preS1 and a titrated amount of 9H9 (low, $1/10^6$; high, $1/10^4$) or diluent was added. A third incubation was performed with diluent or a fixed amount of HBs.OT40 (low, $1/10^6$; high, $1/10^4$). Finally, conjugates were applied as indicated. Chimpanzee pre-treatment serum, dilution 1/16: A_{450} 0·083 with conjugated anti-mouse Ig; A_{450} 0·975 with conjugated HBs.OT42. Anti-HBs, low and high dilutions from stock solution. Results in absorbance at 450 nm.

2nd Incubation				
	9H9 Concn			4th Incubation
Diluent	Low	High	3rd Incubation	Conjugate
1·319	1·550	1·824	Diluent	Anti-human Ig
0·931	0·718	0·137	Low HBs.OT40 conc	Anti-mouse Ig
1·770	1·693	1·060	High HBs.OT40 conc	Anti-mouse Ig

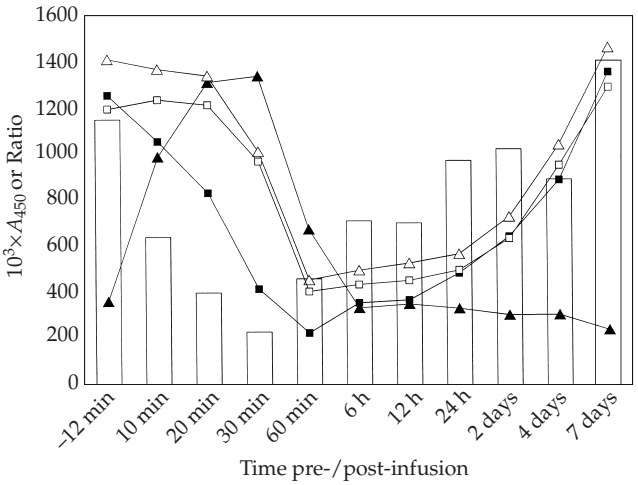

Fig. 3. The residual amount of preS1-Ag-containing particles in serum samples from a chimpanzee obtained during and after administration of a mixture of human monoclonal antibodies was titrated with conjugated anti-S (HBs.OT40, ■; HBs.OT42, □), anti-preS2 (OTB-anti-preS2, △) and anti-human Ig (▲), simultaneously. The bars represent the ratios of the absorbance signals of conjugated HBs.OT40 and HBs.OT42.

2 (curve a) illustrates the reduction in absorbance with dilution of HBs.OT40 in the first anti-HBs incubation step. The amount of HBs.OT40 was measured with anti-mouse Ig conjugate after rinsing the wells and applying a fixed amount of 9H9 to each of the wells. Fig. 2 (curve d) shows that the absorbance of 9H9 as measured with anti-human Ig increases in parallel with the reduction of the amount of mouse HBs.OT40 (anti-S). Comparable results were obtained with incubation of HBs.OT40 (fixed amount) and 9H9 (titrated amount) in the reverse order (curves b, c).

Experiments were repeated on a small scale with the chimpanzee pre-treatment HBsAg. The summary in Table 1 shows that increasing the amount of 9H9 on the chimpanzee pre-treatment HBsAg on solid phase sheep anti-preS1 reduced the possibility of binding mouse HBs.OT40 (anti-S) antibodies. The reverse experiment, loading the chimpanzee HBsAg with a titrated amount of mouse HBs.OT40 (anti-S) and subsequently loading with a fixed amount of 9H9, confirmed these results. These experiments suggest that human 9H9 and mouse HBs.OT40 (anti-S) bind to the same epitope region of the chimpanzee HBsAg.

PreS1-Ag titration by HBs.OT42 (anti-S) and HBs.OT40 (anti-S) conjugates in the chimpanzee serum samples

Chimpanzee serum samples were assayed by means of the sheep anti-preS1 solid phase EIA using the two mouse anti-S conjugates HBs.OT40 (anti-S) and HBs.OT42 (anti-S) in parallel. Mouse OTB-anti-preS2 conjugate was included as a control. Fig. 3 shows that HBsAg measured with the conjugated HBs.OT40 (anti-S) decreased from the start of the infusion, in contrast to the similar signals obtained at −12, 10 and 20 min with conjugated HBs.OT42 (anti-S) or anti-preS2. The lowest ratio of HBs.OT40 to HBs.OT42 was found with the 30 min sample. This time-point coincides with the highest human Ig signal. These results suggest that the binding of HBs.OT40 conjugate to the chimpanzee HBsAg is restricted due to the presence of immune-complexed 9H9 in the same epitope area.

Dissociation by acid treatment of the chimpanzee HBsAg from complexes with human monoclonal anti-HBs

To confirm the presence of HBsAg–human monoclonal anti-HBs immune complexes the chimpanzee serum samples were treated according to the acid immune complex dissociation method of Nishanian *et al*. (1990).

As a pilot experiment artificial immune complexes of HepG2 2.2.15 HBsAg and 9H9 were prepared. HepG2 2.2.15 HBsAg is devoid of specific/non-specific reactive human Ig in contrast to the chimpanzee pre-treatment HBsAg. Indeed, close to the IC_{50} concentration of 9H9 for HepG2 2.2.15 HBsAg, a two- to threefold increase in HBsAg signal was observed after acid treatment compared to non-treated samples. These results were obtained with solid phase mouse monoclonal HBs.OT40 (anti-S) with a restricted binding capacity for HBsAg–9H9 complexes due to shielding of the HBs.OT40 epitope by 9H9. Mouse monoclonal HBs.OT42 (anti-S) was used as conjugate. The presence of human Ig on

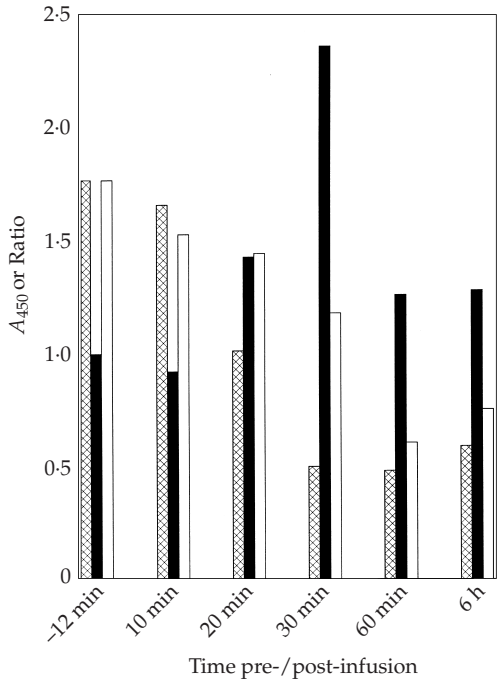

Fig. 4. Chimpanzee serum samples before and after infusion of human monoclonals 9H9 and 4-7B were subsequently incubated with 0·5 M HCl and 0·5 M NaOH to liberate HBsAg from immune complexes. Samples were assayed in EIA with a mixture of mouse anti-HBs monoclonals (HBs.OT20, HBs.OT13 and HBs.OT16; abbreviated as HBs.OT3M) as solid phase and mouse monoclonal HBs.OT42 (anti-S) as conjugate. Absorbance before treatment (cross-hatched bars); after treatment (unfilled bars); and ratio of the signals after/before treatment (solid bars).

HepG2 2.2.15 after pre-incubation with 9H9 was confirmed with anti-human Ig conjugate.

To avoid competition between human 9H9 and solid phase mouse monoclonal HBs.OT40 (anti-S) in chimpanzee samples with suspected HBsAg–9H9–4-7B complexes the solid phase HBs.OT40 (anti-S) was replaced by a combination of three other mouse monoclonal antibodies with anti-S specificity (HBs.OT3M). Fig. 4 shows that after HCl treatment the ratio of treated/untreated HBsAg for chimpanzee samples varied with a maximum deviation at 30 min after the start of the infusion. This is in accordance with results for human Ig on HBsAg particles, as detected by solid phase anti-S and anti-preS1 (Fig. 3).

Detection of 4-7B in immune complexes with chimpanzee HBsAg

By standard pepscan procedures the linear epitope (4-7B peptide) corresponding to the 4-7B antibody was found at the C-terminal end of the S protein (W. P. Paulij and others, unpublished data). When used as a solid phase peptide or as a conjugate it could react with conjugated 4-7B antibody and solid phase 4-7B antibody, respectively, in our EIA protocol. Unfortunately, solid phase peptide 4-7B could not bind the suspected HBsAg–4-7B complex (chimpanzee, 30 min sample). In the reverse experiment, solid phase HBs.OT3M (anti-S) could bind 9H9/4-7B complexed HBsAg (30 min sample) but 4-7B could not be detected with the peptide conjugate. Chimpanzee HBsAg (168 h sample) was loaded on the HBs.OT3M (anti-S) plate and subsequently incubated with 4-7B antibodies. Both HBsAg and 4-7B were used in titrated amounts (twofold dilutions 1/25–1/100 and 1/2–1/32, respectively). These artificial HBsAg–4-7B complexes bound to solid phase anti-S and could be detected with our 4-7B peptide conjugate. Thus, it seems that in the samples obtained shortly after 9H9/4-7B administration chimpanzee HBsAg–4-7B complexes were not present in sufficient amounts to be detected by the 4-7B peptide conjugate.

Finally, the consecutive chimpanzee samples were assayed using solid phase HBs.OT3M (anti-S) and conjugated HBs.OT42 (anti-S) in comparison to the combination of solid phase 4-7B and conjugated 4-7B. Although it was expected that in the latter assay binding of the HBsAg–4-7B complex to solid phase 4-7B and 4-7B conjugate would be inhibited the most, the signal ratio for the two protocols revealed the highest reduction was only 7% for the 30 min sample.

Binding of preS1-Ag in EIA inhibited by 9H9 and 4-7B

As an alternative for the still cumbersome experiments to detect neutralization of HBV in tissue cultures, we assayed the inhibitory effect of 9H9 and 4-7B in an *in vitro* assay using solid phase anti-preS1. This system may resemble the adherence of HBV to human hepatocytes by means of preS1 epitopes.

Experiments with chimpanzee preS1–HBsAg did not yield complete inhibition at very high levels of 9H9 or even 4-7B. This may be explained by the relatively large amounts of S epitopes compared to preS1 epitopes in a sample without HBV DNA-positive particles. Therefore, we changed to the HBV particle-rich reference serum (Mat.O). The specificity of preS1-Ag binding was confirmed by the complete loss of signal from HBsAg in the anti-preS1 assay but not in the HBsAg assay (solid phase HBs.OT3M), after treatment of preS1–HBsAg with trypsin (results not shown) (Itoh & Fujisawa, 1986; Petit *et al.*, 1987). In both assays mouse monoclonal anti-S (HBs.OT42) was used as conjugate.

Both 4-7B and polyclonal human anti-HBs (Hepatect; results not shown) were able to reduce the binding of HBsAg particles to solid phase anti-S (Fig. 5*a*) and solid phase anti-preS1 (Fig. 5*b*) by more than 97%. Similarly, 9H9 could inhibit the binding of HBsAg particles to solid phase anti-S by about 70% and to solid phase anti-preS1 by about 60%. The IC_{50} increased three- to fivefold from the preS1 assay to the S assay. This may be explained by the higher input of HBsAg needed for the preS1 assay and the binding of antibodies to HBsAg particles without preS1-Ag. Experiments were repeated with HepG2 2.2.15 HBsAg to exclude an influence of native human

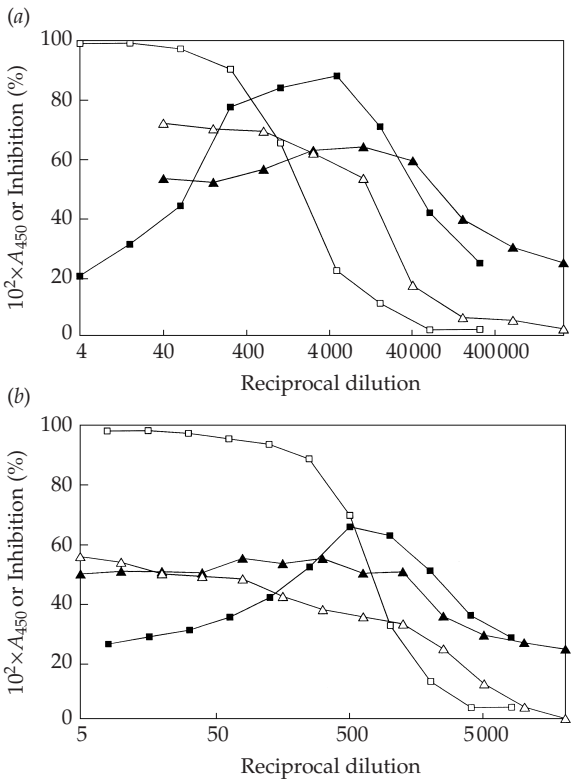

Fig. 5. Inhibitory activity of human monoclonals 9H9 (△) and 4-7B (□) in 'inhibition in solution' assays with solid phase anti-S (HBs.OT3M) (*a*) and anti-preS1 (*b*). HBsAg/S/preS1 from the reference sample (Mat.O) was pre-incubated with titrated amounts of 9H9 or 4-7B. The residual amount of HBsAg was monitored with conjugated anti-S (HBs.OT42). Human Ig on the surface of these residual particles bound to solid phase anti-S/preS1 was monitored with an anti-human Ig conjugate (▲, 9H9; ■, 4-7B). Results in absorbance at 450 nm. (*a*) 50% inhibitory dilution 4-7B, 1/2048; 9H9, 1/20480. (*b*) 50% inhibitory dilution 4-7B, 1/768; 9H9, 1/3840 (50% from maximum of inhibition in this experiment).

Ig normally present on HBsAg particles. The incomplete inhibition by 9H9 in the preS1 assay was confirmed.

Furthermore, monitoring of human Ig (HBsAg-complexed 9H9 or 4-7B) in the same experiments (Fig. 5) indicated that 4-7B prevents the adherence of HBsAg particles to solid phase anti-S as well as anti-preS1 (return to baseline signal at full inhibition). In contrast, a relatively stable amount of 9H9 remained associated with the HBsAg particles at saturation levels of 9H9.

This phenomenon was investigated more directly by using 4-7B and 9H9 as solid phase anti-HBs in EIAs. Post-treatment chimpanzee HBsAg (168 h) was incubated with 9H9 or 4-7B and residual HBsAg assayed on the 4-7B and 9H9 solid phases, respectively. As a reference 9H9 could inhibit chimpanzee HBsAg by as much as 60% on solid phase HBs.OT3M (anti-S). However, 9H9 could inhibit chimpanzee HBsAg by only 25% (dilutions 1/20000 to 1/10) on the 4-7B solid phase. In the reverse experiment, 4-7B could inhibit HBsAg binding to solid phase 9H9 from 20% at a dilution of 1/40000 to 92% at a dilution of 1/4, with a gradual increase over this concentration range. HBsAg detection was always by HBs.OT42 (anti-HBs) conjugate.

These results strongly suggest that 9H9 and 4-7B may have different mechanisms for inhibition or 'neutralization' of HBV particles.

In a separate experiment we investigated the effect of inhibiting the activity of the 1:1 (w/w) mixture of 9H9 and 4-7B on HBsAg (Mat.O) with solid phase mouse monoclonal HBs.OT3M (anti-S) and conjugated mouse monoclonal HBs.OT42 (anti-S). The additive rather than synergistic effect of mixing, as described earlier (Heijtink *et al.*, 1995), was confirmed. Spiking the 9H9 samples with an equal amount of 4-7B resulted in a relatively small change (16%) in the IC_{50} to a higher dilution. However, the inhibition curve showed the 4-7B characteristics and inhibition now reached 99%. The accompanying human Ig curve resembled the 4-7B/human Ig curve. These results suggest that at the level of maximal efficacy of 9H9, spiking 9H9 with 4-7B changes the additive effect of 4-7B to a complementary effect, thereby also changing the type of 'neutralization'.

'Neutralization' of chimpanzee HBsAg by human monoclonal 9H9/4-7B *in vitro* and *in vivo*

In an earlier experiment we found the IC_{50} of 9H9 and 4-7B for human HBsAg to be 110 IU/l and 12 IU/l, respectively. In this *in vitro* assay inhibitory activity occurred for HBsAg at a concentration of 6 ng/ml after incubation of equal volumes. According to our RIA results with chimpanzee HBsAg 50% inhibition was obtained at 110 IU/l and 4 IU/l with 6 ng/ml HBsAg and equal volumes. It may be estimated that the minimum amount of HBsAg in the circulation of the chimpanzee is about 90 mg (36 μg/ml HBsAg in serum, 2·5 l serum).

For 50% inhibition of 90 mg HBsAg about 1650000 IU 9H9 or 300000 IU 4-7B will be needed *in vitro*. The chimpanzee received about 364000 IU of 9H9 and 2200 IU of 4-7B which is about 20% of the amount needed for only 50% inhibition *in vitro* with 9H9 and less than 1% needed for 50% inhibition by 4-7B. This may explain the relatively low concentrations and the absence of detectable 4-7B in HBsAg immune complexes. Furthermore, the reduction by 80% of detectable HBsAg in serum between 30 min and 24 h after the start of the infusion may point to elimination of immune-complexed virus particles rather than shielding of epitopes.

Discussion

About 30 min after the start of the infusion of monoclonal anti-HBs, the amount of detectable HBsAg in chimpanzee blood had reached its lowest level. The pre-treatment level of HBsAg was restored after about 7 days.

This study confirms, although in part by indirect evidence, that human monoclonal anti-HBs administered intravenously

to an HBsAg carrier chimpanzee can form immune complexes *in vivo*. The immune complexes could be destroyed by acid treatment.

Based on rough calculations and using *in vitro* inhibition experiments for estimates of *in vivo* results, it is assumed that the amounts of 9H9 and 4-7B administered to the chimpanzee could be responsible for only a small reduction in accessible HBsAg in the circulation. It seems likely that, in addition to covering the 9H9 and 4-7B epitopes on HBsAg, immune complexes containing HBsAg may be eliminated from the circulation by phagocytosis of the immune aggregates by macrophages.

The aim of this study was to learn the fate of 9H9 and 4-7B human monoclonal antibodies in the circulation of a chronically infected chimpanzee in the first hours and days after administration. For the linear epitope of the human monoclonal 4-7B it was relatively easy to select a corresponding peptide for specific detection of 4-7B in immune complexes. However, probably due to the low concentration of 4-7B, we were not able to detect HBsAg-complexed 4-7B in chimpanzee serum with conjugated 4-7B peptide nor were we able to bind the presumed HBsAg–4-7B immune complexes to solid phase 4-7B peptide. Artificial complexes with high loads of 4-7B were positive with the 4-7B peptide conjugate in the same assay.

By chance, one of our mouse anti-HBs monoclonal antibodies (HBs.OT40) was found to compete with 9H9 for the same epitope region of HBsAg. Using HBs.OT40 specificity and assuming that *in vitro* and *in vitro* processes are alike, we tested the accessibility of the 9H9 epitope region on chimpanzee HBsAg during 9H9/4-7B treatment. The results suggest that up to 30 min after the start of the infusion there is reduced HBsAg activity with HBs.OT40 (anti-S) conjugate compared to HBs.OT42 (anti-S) conjugate due to loading of HBsAg in the circulation by 9H9. At 30 min after the start of the infusion the level of HBsAg was minimal, as assayed by solid phase anti-HBs or anti-preS1 and conjugated anti-S or anti-preS2. The anti-human Ig signal confirmed maximum complexing of HBsAg with 9H9/4-7B in the 30 min sample. Furthermore, the experiment with solid phase anti-preS1 and conjugated anti-preS2 strongly suggested that HBsAg particles were removed from the circulation and not just covered with 9H9/4-7B in view of similar findings for solid phase anti-S and anti-preS1.

Earlier experiments on 'inhibition in solution' of HBsAg by 9H9 had already revealed that this human monoclonal antibody could not completely inhibit the binding of HBsAg to solid phase anti-HBs in contrast to human monoclonal 4-7B. The use of solid phase anti-preS1 with anti-preS2 detection confirmed the deviant behaviour of 9H9 once more. Even extremely large amounts of 9H9 did not change the rate of inhibition of binding to solid phase anti-S markedly (Fig. 5 *a*). The lowest rate of inhibition (20%) by titrated amounts of 9H9 was observed for chimpanzee HBsAg measured on solid phase 4-7B. In the reverse experiment, inhibition by 4-7B and detection on solid phase 9H9, more than 90% inhibition was found. Could it be that 4-7B is more effective in the aggregation of particles and 9H9 in complete shielding?

Our observations stress the need for models based on infectivity assays, whether in tissue cultures or in animals. From this chimpanzee study we have learned that monoclonal anti-HBs, and probably polyclonal anti-HBs as well, may help to eliminate virus particles from the circulation without completely blocking or shielding the main epitopes used for detection of HBsAg in diagnostic assays.

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