EXPERIMENTAL and MOLECULAR MEDICINE, Vol. 40, No. 1, 145-149, February 2008

In vivo hepatitis B virus-neutralizing activity of an anti-HBsAg humanized antibody in chimpanzees

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Accepted 7 January 2008

Abbreviations: anti-HBc, anti-HBcAg; anti-HBs, anti-HBsAg CDRs, complementarity determining regions; FRs, framework regions; HBIG, hepatitis B immune globulin; HBcAg, hepatitis B virus core antigen; HBsAg, hepatitis B virus surface antigen

Abstract

Previously, we constructed a humanized antibody (HuS10) that binds to the common *a* antigenic determinant on the S protein of HBV. In this study, we evaluated its HBV-neutralizing activity in chimpanzees. A study chimpanzee was intravenously administered with a single dose of HuS10, followed by intravenous challenge with the *adr* subtype of HBV, while a control chimpanzee was only challenged with the virus. The result showed that the control chimpanzee was infected by the virus, and thus serum HBV surface antigen (HBsAg) became positive from the 14th to 20th week and actively acquired serum anti-HBc and anti-HBs antibodies appeared from the 19th and 23rd week, respectively. However, in the case of the study chimpanzee, serum HBsAg became positive from the 34th to 37th week, while actively acquired serum anti-HBc and anti-HBs antibodies appeared from the 37th and 40th week, respectively, indicating that HuS10 neutralized the virus *in vivo* and thus delayed the HBV infection. This novel humanized antibody will be useful in the immunoprophylaxis of HBV infection.

Keywords: antibodies, monoclonal; hepatitis B surface antigens; hepatitis B virus; Pan troglodytes; immunization, passive; immunotherapy

Introduction

HBV infection is a worldwide public health problem affecting 300 million persistent chronic carriers having a high risk to develop hepatocellular carcinoma (Tiollais and Buendia, 1991). For the prevention or post-exposure prophylaxis of HBV infection, hepatitis B immune globulin (HBIG) prepared from pooled human anti-HBsAg plasma is administered to infants born of HBsAg-HBeAg positive mothers at birth, susceptible individuals with an acute exposure to infectious HBV-containing material or orthotopic liver transplant patients with chronic HBV-related liver disease (Beasley *et al*., 1983; McGory *et al*., 1996; Terrault *et al*., 1996; Sawyer *et al*., 1998). However, the currently available HBIG is not an ideal source of antibody due to the limited availability and low specific activity. Therefore, mAbs specific to the surface antigens of HBV would be a good alternative for the immunoprophylaxis of HBV infection.

The HBV envelope contains three related surface glycoproteins called the large (L), middle (M), and small (S) proteins. All these proteins are the product of a single open reading frame that is divided into the preS1, preS2, and S regions (Heermann *et al*., 1984). The S protein is encoded by the S region. The M protein contains the preS2 and S regions, and the L protein contains the preS1, preS2, and S regions. The S protein carries the common *a* determinant and two sets of mutually exclusive subtype determinants *d/y* and *w/r*, and thus the four major subtypes (*adw*, *adr*, *ayw*, and *ayr*) of the HBV surface antigen (HBsAg) denote the antigenic types of HBV (Courouce *et al*., 1983). The serotypes have an unequal distribution worldwide. The *y* and *r* determinant are absent from Far-East Asia and from Africa, respectively. The common *a* determinant of the S protein was shown to elicit virus- neutralizing and protective antibodies (Bhatnager *et al*., 1982; Dreesman *et al*., 1982; Heermann *et al*., 1987). In addition, a single mAb specific to the common *a* determinant of the S protein was demonstrated to have virus-neutralizing activity in chimpanzees or

humans (Ogata *et al*., 1993; Heijtink *et al*., 1999; Eren *et al*., 2000; Galun *et al*., 2002). However, since the escape mutants of the common *a* determinant have arisen (Fujii *et al*., 1992; McMahon *et al*., 1992; Kohno *et al*., 1996; Terrault *et al*., 1998), development of more new anti-HBV neutralizing antibodies would be beneficial in the immunoprophylaxis of HBV infection.

Murine mAbs are easy to produce, but their therapeutic use in humans is limited because of human anti-mouse antibody (HAMA) response during treatment (Shawler *et al*., 1985). To circumvent the problem, humanized antibodies have been constructed by grafting the mouse complementarity determining regions (CDRs), which form antigen-binding pocket, onto homologous human antibody variable regions, while retaining some murine residues in framework regions (FRs) that are predicted to influence the conformation of CDRs (Riechmann *et al*., 1988; Queen *et al.,* 1989; Nakatani *et al.,* 1994). Several humanized antibodies are in clinical use in humans (Reichert *et al*., 2005).

Previously, we generated a murine mAb, H67 that recognizes the *a* determinant on the S protein (Ryu *et al*., 1994) and subsequently developed a humanized antibody (HuS10) of which affinity is same as that of the parental murine mAb (Ryu *et al.,* 1996). The antibody showed neutralizing activity against both the *adr* and *ayw* subtypes of the virus in an *in vitro* infection of adult human hepatocyte primary culture by HBV (Ryu *et al*., 1996). In this study, we demonstrated *in vivo* HBV-neutralizing activity of HuS10 in chimpanzees.

Materials and Methods

Cell culture

The dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cell line, DG44 was used for stable expression of the humanized antibody. The DG44 cells were grown at 5% CO_2, 37°C in DMEM/F12 (GIBCO/BRL) supplemented with hypoxanthine (10 mg/l), thymidine (10 mg/l), glycine (50 mg/l), glutamine (587 mg/l), glucose (4.5 mg/l), 10% FBS, and Antibiotics-Antimycotics (GIBCO/BRL).

Production of humanized antibody

The humanized heavy and light chain expression plasmids were cotransfected into DHFR-deficient CHO cell line (DG44), and stably transformed cell lines were selected in a medium containing G418 (550 μg/ml) and subsequently subjected to methotrexate (MTX) selection for gene amplification, as described previously (Ryu *et al*., 1996). A recombinant CHO cell line secreting the humanized antibody was grown in serum free medium (CHO-S-SFMII, GIBCO/BRL), and the culture supernatant was subjected to affinity chromatography on Protein G-Sepharose 4B column (Pharmacia), as described previously (Ryu *et al.,* 1996).

Chimpanzee study

Two chimpanzees (CA0218 and CA0215), which were born in a breeding colony in the United States (White Sands Research Center, Alamogordo, NM) under a Food and Drug Administration contract, were used in this study. These animals had not been previously inoculated with any HBV-containing materials and were seronegative for all HBV-associated serological markers. The study chimpanzee (CA0215) was intravenously administered with purified HuS10 at a dose of 5 mg/kg (body weight), while the control animal (CA0218) was administered with PBS. Three days later, 10^{-5} dilution of human sera containing *adr* subtype of wild type HBV (kindly provided by Dr. R. H. Purcell at NIH), whose end-point infectivity titer in chimpanzees was previously determined to be 10^{-8} (Tabor *et al*., 1983), was inoculated intravenously into the animals. Serum samples taken from the chimpanzees were monitored for levels of the humanized antibody HuS10, HBsAg, and antibodies to HBcAg and HBsAg as well as for serum chemistry, hematology, and urinanalysis. Serological assays of the HBV antigens and anti-HBV antibodies were carried out using commercial kits (Abbott Laboratories, North Chicago, IL) in WSRC.

Results

In vivo HBV-neutralizing activity of humanized antibody HuS10 in chimpanzees

Previously, we generated a murine mAb (H67) that binds to both *adr* and *ayw* subtypes of HBsAg, and subsequently constructed a humanized antibody (HuS10) whose affinity (5×10^8 M^{-1}) is same as that of the parental murine mAb (Ryu *et al*., 1996). HuS10 neutralized both the *adr* and *ayw* subtypes of HBV in an *in vitro* infection of adult human hepatocyte primary culture by HBV (Ryu *et al*., 1996).

To evaluate the virus-neutralizing activity of HuS10 in chimpanzees, a stable CHO cell line expressing HuS10 was grown in serum-free medium and the antibody was purified from the culture supernatant by an affinity chromatography on Pro-

Table 1. The analysis of HBV infection in the serum of the control and the study chimpanzees.

A. Detection of HBsAg

			Study week											
			1-13	14	15	16	17	18	19	20	21-29	30	34	37	40-52
I	Control	CA0218	N	R	R	R	R	R	R	R	N	N	N	N	N
II	HuS10	CA0215	N	N	N	N	N	N	N	N	N	N	R	R	N

Titers were determined using a commercial kit (Abbott Laboratories). The positivity was defined as the absorbance of the negative control mean plus the factor of 0.05 according to the manufacturer's instruction manual. N = Non-reactive and R = Positive reactivity.

B. Detection of anti-HBc antibodies

			Study week											
			1-18	19	20	21	22	23	24	25	26	27-30	34	37	40-52
I	Control	CA0218	N	R	R	R	R	R	R	R	R	R	R	R	R
II	HuS10	CA0215	N	N	N	N	N	N	N	N	N	N	N	R	R

Titers were determined using a commercial kit (Abbott Laboratories). Specimens with absorbance values equal to or lower than the cutoff value are considered reactive according to the manufacturer's instruction manual. Cutoff value was determined using the following equation: 0.4 (NCx) + 0.6 (PCx) = Cutoff Value. N = Non-reactive and R = Positive reactivity.

C. Determination of anti-HBs antibodies

			Study week													
			1-22	23	24	25	26	27	28	29	30	34	37	40	43	46	49-52
I	Control	CA0218	0	16	24	30	38	51	62	130	> 150	> 150	> 150	> 150	> 150	> 150	> 150
II	HuS10	CA0215	0	0	0	0	0	0	0	0	0	0	0	15	36	133	> 150

Titers were determined using a commercial kit (Abbott Laboratories). Result of < 5 was considered 0.

Table 2. The titers of administered HuS10 in chimpanzees.

			Study week										
			-4	-2	1 (Day 4)	2	3	4	5	6	7	8	9	10
I	Control	CA0218	0	0	0	0	0	0	0	0	0	0	0	0
II	HuS10	CA0215	0	0	144	60	34	16	27	11	0	0	0	0

Titers were determined using a commercial kit (Abbott Laboratories). Result of < 5 was considered 0.

tein G-Sepharose, as described in the Materials and Methods. The purified antibody was 99% pure and free of endotoxin and pyrogen, and did not exhibit abnormal toxicity (data not shown).

Two chimpanzees, one study chimpanzee and one control chimpanzee, were used in this study. The study chimpanzee (CA0215) was intravenously administered with the humanized antibody (5 mg/kg) and three days later the animals were challenged with 10^{-5} dilution of human sera containing *adr* subtype of HBV, whose end-point infectivity titer in chimpanzees had been determined to be 10^{-8} (Tabor *et al*., 1983). The control chimpanzee (CA0218) was only challenged with the same dose of the virus. The course of HBV infection in the chimpanzees was weekly monitored by serological and biochemical analyses of the sera for 1 yr (Table 1). Also, the titers of the administered humanized antibody HuS10 in the sera of chimpanzees were measured for the first 10 weeks (Table 2).

In the case of the control chimpanzee, serum HBsAg became positive from the 14th to 20th week. Also, actively acquired serum anti-HBc and anti-HBs antibodies appeared from the 19th and 23rd week, respectively and remained at elevated level through the end-point of this experiment. In contrast, in the case of the study chimpanzee, serum HBsAg became positive from the 34th to 37th week, while actively acquired serum anti-HBc and anti-HBs antibodies appeared from the 37th and 40th week, respectively and remained at elevated level through the end-point of this experiment. The courses of HBV infection in the chimpanzees are summarized in Figure 1. The results indicate that the humanized antibody neutralized the virus *in vivo* and thus protected the chimpanzee from HBV infection.

The HuS10 antibody administered in the study chimpanzee was detected in the serum for 6

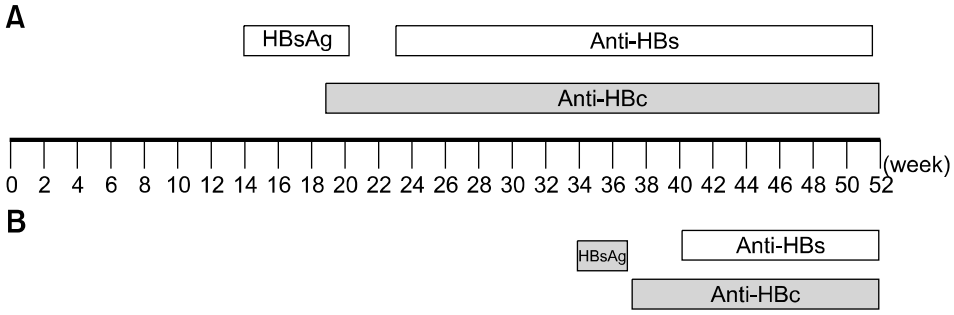

Figure 1. The course of HBV infection showing the appearance of HBsAg and anti-HBV antibodies in the serum of the control chimpanzee (A) and the study chimpanzee (B).

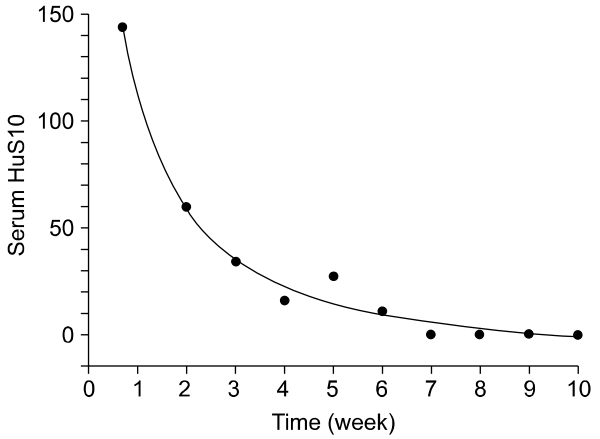

Figure 2. The serum levels of the administered HuS10 in the study chimpanzee. The study chimpanzee (CA0215) was intravenously administered with purified HuS10 at a dose of 5 mg/kg (body weight) and three days later, *adr* subtype of wild type HBV was inoculated intravenously. The titer of HuS10 was measured using the commercial kit (Abbott Laboratories).

weeks (Table 2). The humanized antibody had a half-life of approximately 8 days in the challenged chimpanzee (Figure 2), which is shorter compared to that (2-3 weeks) of human IgG in unchallenged chimpanzees or humans (Reichert *et al.*, 2005). This may be because the humanized antibody molecules bound to the challenged HBV and were eliminated from the blood of the challenged chimpanzee.

Discussion

For the prevention or post-exposure prophylaxis of HBV infection, human mAbs specific to the common *a* determinant of the S protein have been developed and their HBV-neutralizing activities were demonstrated in chimpanzees or humans (Ogata *et al.*, 1993; Heijtink *et al.,* 1999; Eren *et al.*, 2000; Galun *et al.,* 2002). However, mostly their HBV-neutralizing activities were demonstrated against only *ayw* subtype. In addition, considering that the common *a* determinant region of the S protein has highly conformational structure and the escape mutants of the common *a* determinant have arisen (Fujii *et al.,* 1992; McMahon *et al.,* 1992; Bruce and Murray, 1995; Howard, 1995; Kohno *et al.,* 1996; Keum *et al.*, 1998; Terrault *et al.*, 1998), development of more new HBV-neutralizing antibodies is beneficial in the immunoprophylaxis of HBV infection. In this study, we demonstrated the HBV-neutralizing activity of the humanized antibody (HuS10) against the *adr* subtype (Figure 1). This humanized antibody may be useful in the immunoprophylaxis of HBV infection in the Far-East Asia, where the *ad* subtype is endemic.

Acknowledgments

We thank Dr. Robert Purcell at NIH for providing the wild type HBV and Taehyoung Kwon for editorial assistance. This work was supported by Ministry of Health and Welfare Grant A050260 and Korea Research Institute of Bioscience and Biotechnology Research Initiative Program Grant KGM3100612.

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