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Original Article

Effect of Ethanol on Antigenicity of Hepatitis B Virus Envelope Proteins

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SUMMARY: Hepatitis B e antigen-positive human serum was treated with 50-90% ethanol at room temperature for 1-60 min, then the antigenicity of S antigen (hepatitis B surface antigen, in a narrow sense) was determined by radioimmunoassay and the antigenicities of pre-S1 and pre-S2 antigens were measured by enzyme immunoassay. In addition, hepatitis B virus (HBV) DNA in the treated serum was detected by polymerase chain reaction. All antigenicities markedly decreased within 60 min at an ethanol concentration of 70-80%, and the decrease was faster in pre-S1 and pre-S2 antigens than in S antigen. Although HBV DNA remained in all ethanol-treated serum samples, no HBV DNA was detected after treatment with 1% sodium hypochlorite for 1 min. Based on the results, we speculate that one mechanism of loss of HBV infectivity by ethanol is the inhibition of virus binding to hepatocytes.

INTRODUCTION

A culture system has not yet been established investigating in detail the inactivation of hepatitis B virus (HBV) by disinfectants. It has been necessary to demonstrate the loss of infectivity by chimpanzee experiments or to confirm the complete disappearance of HBV DNA in vitro. Although Kobayashi et al. (1) reported that ethanol eliminated HBV infectivity in chimpanzees, the effect has not been recognized amply in clinical practice, and the World Health Organization (WHO) has acknowledged only glutaraldehyde and sodium hypochlorite as effective disinfectants for HBV (2). Several years ago, it was shown that the HBV surface antigen consists of S, pre-S1, and pre-S2 antigens, and that pre-S1 and pre-S2 antigens are closely involved in the process of binding to hepatocytes (3-7). Although various parameters of the effects of disinfectants for HBV have been investigated (8-11), there have been no reports regarding their effect on pre-S1 and pre-S2 antigens. In this study, we investigated the effect of ethanol on HBV surface antigens, including measurement of the antigenicities of pre-S1 and pre-S2 antigens, by enzyme immunoassay (EIA) in order to clarify the mechanism of loss of HBV infectivity by ethanol.

MATERIALS AND METHODS

Test serum: Human serum from a hepatitis B e (HBe) antigen-positive HBV carrier (hepatitis B surface [HBs] antigen in hemagglutination titers; 2^{15}, Reversecell®; YAMANOUCHI Co., Ltd., Tokyo) was used. The serum was diluted 5 or 10 times with sterilized physiological saline and used as the serum samples.

Disinfectants: Dehydrated ethanol (YOSHIDA Pharmaceutical Co., Ltd., Tokyo, Lot. 394411) and 6% sodium hypochlorite (YOSHIDA Pharmaceutical, Lot. 099660) were diluted with sterile purified water.

Measurement of antigenicities of S and pre-S2 antigens using commercial assay kits: To a 1.5-ml microtube, 900 μl of disinfectant preparation and 100 μl of sample were added and mixed well, then reacted at room temperature. After being reacted for a specified time, 100 μl of the mixture treated with ethanol and 100 μl of that treated with sodium hypochlorite were neutralized (in the case of ethanol, neutralization means dilution) by 10-fold dilution with 900 μl of sterilized physiological saline or with 900 μl of sterilized 0.5% sodium thiosulfate, respectively. Changes in antigenicity were measured in the neutralized solutions. The antigenicity of the S antigen was determined using Ausria ®II-125 (DAINABOT Co., Ltd., Tokyo) and that of pre-S2 antigen was measured using HBV Genotype EIA (Institute of Immunology Co., Ltd., Tokyo) developed for HBV genotype determination.

Measurement of antigenicities of pre-S1 and pre-S2 antigens by EIA systems established in our laboratory: Monoclonal antibody (Institute of Immunology) (12-14) as the first antibody (No. T0606 and No. 5521) and as the secondary antibody (No. T0606 and No. 5520), labeled with horseradish peroxidase, were in the antigenicity assay systems of the pre-S1 and pre-S2 antigens, respectively. The secondary antibody No. T0606 was labeled in our laboratory with enzyme by means of the modified periodic acid method (15).

To a 1.5-ml microtube, 90 μl of ethanol preparation and 10 μl of sample were added and mixed well, then reacted at room temperature. A 10 μl aliquot of the mixture was neutralized by 10-fold dilution with 90 μl of Tris hydrochloride buffer (20 mM, pH 7.5) supplemented with 10% fetal calf serum (Tris-HCl-10% FCS) at 1, 2, 5, 10, 30, and 60 min after the initiation of the reaction.

To a 96-well microplate (Greiner GmbH, Frickenhausen, Germany), a 50 μl aliquot of physiological saline containing

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5 μg/ml of monoclonal antibody was added, and the plate was kept at 4°C overnight to immobilize the antibody to its surface. Subsequently, the plate was washed five times with physiological saline containing 0.05% Tween 20 (washing solution). The sample was treated with ethanol and neutralized, then diluted 1-8 times with Tris-HCl-10% FCS, after which 50 μl was added to the antibody-immobilized well. The plate was shaken at room temperature for 2 h (first reaction) and washed with washing solution. Next, each well received 50 μl of Tris-HCl-25% FCS supplemented with 1% mouse serum containing 3 μg/ml enzyme-labeled antibody, then the solution was shaken at room temperature for 2 h (second reaction). After the plate had been washed with washing solution, 100 μl of reagent for color development (citrate-phosphate buffer containing 0.6 mg/ml O-phenylene diamine, 0.0067 % H_2O_2) was added and reacted in a dark room for 30 min. The reaction was terminated by adding 50 μl of 4 N H_2SO_4, and wells were tested for absorbance at 492 nm. Changes in the antigenicity of ethanol-treated samples were obtained by comparison with the absorbance of diluted non-ethanol-treated samples.

Detection of HBV DNA: Using the nucleic acid extraction reagent SepaGene ®RV-R (SANKO JUNYAKU Co., Ltd., Tokyo), DNA was extracted from the neutralized samples and amplified by polymerase chain reaction (PCR), and bands corresponding to 118 bp and 1,085 bp were detected. The sequences of PCR primers were as follows (16). For the HBV-X region, 5′-GTCCCCTTCTTCATCTGCCGT-3′ (HBx1, sense primer, nt 1487-1507) and 5′-ACGTGCAGAGGTG AAGCGAAG-3′ (HBx2, antisense primer, nt 1604-1584) (118 bases). For HBV-large S (pre-S1+pre-S2+S gene), 5′-TCACCATATTCTTGGGAACAAGA-3′ (P1, sense primer, nt 2817-2839) and 5′-GGCACTAGTAAACTGAGCCA-3′ (S2-2, antisense primer, nt 687-668) (1085 bases).

It was preliminarily confirmed that neutralized disinfectant-treated samples do not affect the measurement antigenicities of S, pre-S1, and pre-S2 antigens, or the detection HBV DNA.

Table 1. Effect of ethanol and sodium hypochlorite on antigenicity of the S antigen

Disinfectant	Concentration (%)[5]	Exposure time(min)	cpm/C.O[6]	judgment[7]
EtOH[1]	90	10	10.6	+
	90	30	5.1	+
	90	60	3.9	△
	80	10	6.6	+
	80	30	2.5	△
	80	60	0.9	–
	70	10	4.5	△
	70	30	1.5	△
	70	60	0.9	–
	50	10	27.0	+
	50	30	29.2	+
	50	60	20.8	+
NaOCl[2]	0.5	1	1.2	△
	0.5	10	0.9	–
	0.25	1	7.0	+
	0.25	10	0.7	–
	0.1	1	26.8	+
	0.1	10	0.9	–
Neutralized EtOH 90 (+)[3]		60	40.6	+
Neutralized NaOC1 0.5 (+)[3]		10	32.2	+
Neutralized EtOH 90 (–)[4]		60	0.5	–

[1] Ethanol.
[2] Sodium hypochlorite.
[3] HBV positive control: A specified amount of sample was added after the disinfectant was neutralized.
[4] HBV negative control: Instead of sample, a specified amount of sterile purified water was added after the disinfectant was neutralized.
[5] EtOH, (vol/vol); NaOCl, (wt/vol).
[6] C.O (Cut-off value): (average cpm of negative control attached to the reagent) × 2.1.
[7] cpm/C.O of 5.0 or higher: positive, +; 1.0-5.0: judgment suspended, △ ; lower than 1.0: negative, –.

RESULTS

Measurement of antigenicities of S and pre-S2 antigens using commercial assay kits: Changes in the antigenicity of S antigen after treatment with disinfectants, determined using Ausria II-125, are shown in Table 1. The antigenicity of S antigen was decreased by ethanol treatment. The most marked decrease was observed at about 70-80% (vol/vol), and the sample was converted to below the cut-off value by treatment for 60 min. The decreases in antigenicity within this concentration range were greater than those at 90%. In contrast, at the level of 50%, antigenicity corresponding to about half that of the positive control (neutralized EtOH 90) persisted even after 60-min treatment.

Samples treated with 0.1-0.5% sodium hypochlorite yielded values below the cut-off after 10-min treatment.

Changes in the antigenicity of pre-S2 antigen after treatment with disinfectants, measured using HBV Genotype EIA, are shown in Table 2. Based on the findings that the test serum reacted with epitopes b, k, and s, the virus was concluded to correspond to genotype C. The reactivity of epitope b, which is common in all HBV genotypes, became below the cut-off value after 1-min treatment, and the reactivities of epitope k and s became below the cut-off value after 10-min treatment, with 80% ethanol. At 50%, these reactivities decreased; the decreases were smaller than those at 80%, and remained even after treatment for 10 min.

In samples treated with sodium hypochlorite, all epitope reactivities showed values below the cut-off after treatment for 1 min at 0.5% and for 10 min at 0.1%, respectively.

Measurement of antigenicities of pre-S1 and pre-S2 antigens by EIA systems established in our laboratory: Changes in the antigenicities of pre-S1 and pre-S2 antigens (absorbance) after ethanol treatment, measured by laboratory-made EIA systems, are shown in Fig. 1. The antigenicities of pre-S1 and pre-S2 antigens were similarly decreased by ethanol treatment, and the decreases at 70-80% were most marked; larger than the decreases at 90%. Within the range from 50 to 70%, the antigenicities decreased in a concentration-dependent manner. At all concentrations, the decrease was dependent on the duration of treatment.

Detection of HBV DNA: The results of HBV DNA detection after disinfectants treatment are shown in Table 3. The HBV DNA amplified using the primers was detected in all ethanol-treated samples regardless of the concentration or duration of treatment. In contrast, no HBV DNA was detected in samples treated with 1% sodium hypochlorite for 1 min.

DISCUSSION

As described in the Introduction, HBV surface antigen

Table 2. Effect of ethanol and sodium hypochlorite on pre-S2 epitope

Agents and concentration	Exposure time(min)	Epitope									
		b		k		s		u		m	
		Abs	Judgment[4]	Abs	Judgment	Abs	Judgment	Abs	Judgment	Abs	Judgment
Physiological saline	10	2.621	+	2.206	+	2.729	+	0.036	–	0.045	–
Neutralized EtOH 80 (+)[1]	60	2.176	+	1.673	+	2.419	+	0.047	–	0.034	–
Neutralized NaOC1 0.5 (+)[1]	10	2.640	+	2.178	+	2.838	+	0.042	–	0.04	–
80% (vol/vol) EtoH	1	0.096	–	0.128	+	0.124	+	0.045	–	0.028	–
80% EtOH	10	0.021	–	0.027	–	0.016	–	0.032	–	0.026	–
50% EtOH	10	0.140	+	0.158	+	0.152	+	0.036	–	0.024	–
50% EtOH	60	0.090	–	0.102	–	0.107	–	0.035	–	0.029	–
0.5% (wt/vol) NaOCl	1	0.018	–	0.016	–	0.006	–	0.027	–	0.027	–
0.1% NaOCl	1	0.017	–	0.608	+	0.009	–	0.026	–	0.027	–
	10	0.017	–	0.016	–	0.009	–	0.032	–	0.026	–
(–) Control[2]		0.017		0.022		0.016		0.034		0.027	
(+) Control[3]		1.091		1.012		1.217		1.024		1.565	

[1] HBV positive control: A specified amount of sample was added after the disinfectant was neutralized.
[2] Negative control attached to the reagent.
[3] Positive control attached to the reagent.
[4] The cut-off value was set to (Abs + 0.1) of the negative control of each epitope, and values at or higher than the cut-off value were judged as positive, +, and values lower than the cut-off were judged as negative, –.

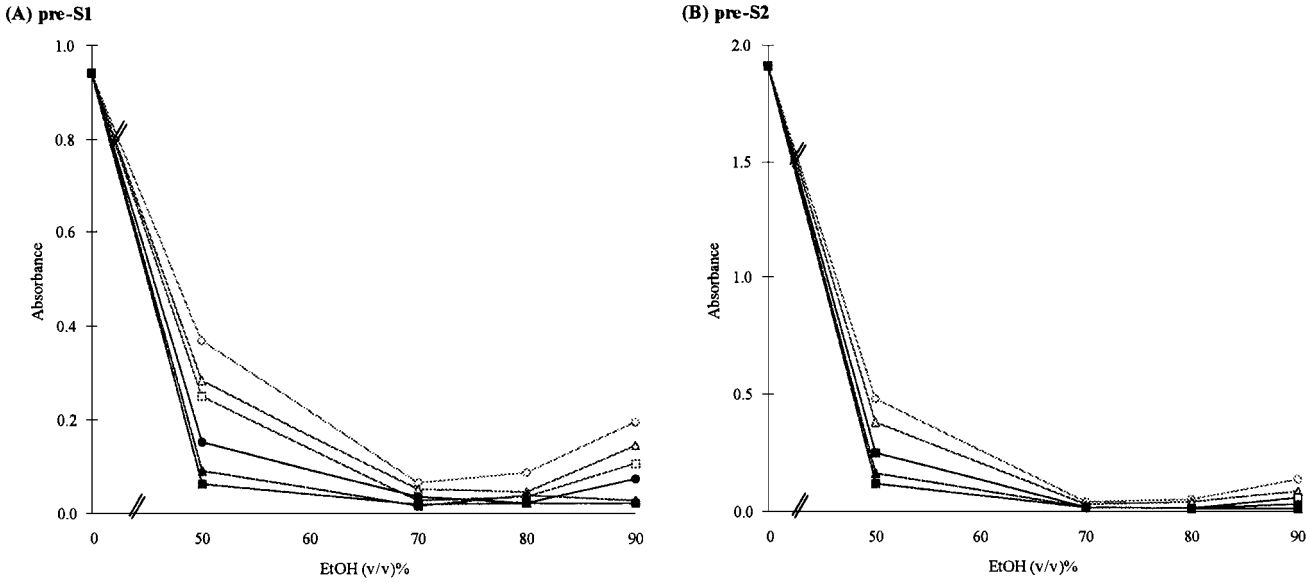

Fig. 1. Changes in the antigenicities of pre-S1 (A) and pre-S2 (B) antigens after ethanol treatment.
○ 1 min , □ 2 min, △ 5 min, ● 10 min, ▲ 30 min, ■ 60 min.

consists of S, pre-S1, and pre-S2 antigens, and the pre-S1 and pre-S2 antigens have been shown to play an important role in the attachment of HBV to hepatocytes (3-7). Blockage of virus attachment to the target cells is a general measure for the prevention of virus infection including HBV infection (17). In this study, to investigate the effect of ethanol on the binding of HBV to hepatocytes, we measured the antigenicity of pre-S2 using a commercial assay kit for genotype determination and the antigenicities of pre-S1 and pre-S2 antigens using laboratory-made assay systems. Antigenicity of S antigen (HBs antigen, in a narrow sense) was simultaneously determined by radioimmunoassay (RIA), which is considered an adequate method for evaluating the effects of disinfectants on HBV.

As described in the Results, treatment of no less than 60 min with 80% ethanol was required to convert the antigenicity of S antigen to below the cut-off value on RIA. Epitope b, which is common in all HBV genotypes, was converted by 1-min treatment to below the cut-off value on the measurement of antigenicity of pre-S2 antigen using a commercial assay kit, and the antigenicities of pre-S1 and pre-S2 antigens after 1-min treatment were lower than 10% of the positive control activity on measurement by laboratory-made EIA using monoclonal antibodies. According to an experimental infection with 80% ethanol treatment in chimpanzees, performed by Kobayashi et al. (1), infectivity was lost by only 2-min treatment . These results have suggested that one mechanism of loss of HBV infectivity by ethanol is based on the inhibition of virus binding to hepatocytes.

The ethanol concentration used for disinfection was 70-80%, and the bactericidal effect decreases at 90% or higher

Table 3. Effect of ethanol and sodium hypochlorite on HBV DNA

Agents	Concentration (%)[3]	Exposure time (min)	PCR products[4] 118 bp	PCR products[4] 1085 bp
EtOH	90	10	+	+
	90	30	+	+
	90	60	+	+
	80	10	+	+
	80	30	+	+
	80	60	+	+
	70	10	+	+
	70	30	+	+
	70	60	+	+
	50	10	+	+
	50	30	+	+
	50	60	+	+
NaOCl	1	1	–	–
	1	10	–	–
Neutralized EtOH 90 (+)[1]		60	+	+
Neutralized NaOC1 1 (+)[1]		60	+	+
Neutralized EtOH 90 (–)[2]		60	–	–

[1] HBV positive control: A specified amount of sample was added after the disinfectant was neutralized.
[2] HBV negative control: Instead of sample, a specified amount of sterile purified water was added after the disinfectant was neutralized.
[3] EtOH, (vol/vol); NaOCl, (wt/vol).
[4] HBV DNA positive, + ; negative, –.

(18). Yamashita hypothesized that the cell membrane and protein structure of microorganisms are rapidly degenerated or destroyed in an ethanol concentration range of 40-80%, but that the ethanol structure changes at 90% or higher so that the hydrophobic regions are gently pulled apart from each other, becoming less effective for microorganisms (19). The effect of ethanol for reducing the HBV surface antigenic activity was most marked at about 70-80%, which range of concentration is also most effective for reducing the bactericidal activity, and was higher than the effect at 90%. The HBV surface antigen is a protein with a complex composition and tertiary structure, and degeneration caused by cleavage of hydrogen bonds in the protein (20) is considered to be an antigenic activity-reducing mechanism of ethanol.

On confirmation of HBV DNA by PCR, no effect of ethanol on the nucleic acid reacting with the primers was observed, as was expected, indicating that the ethanol does not destroy HBV DNA.

On the other hand, ethanol has been reported to decrease the activity of HBV DNA polymerase (10), though it is not yet clear whether the DNA polymerase is an essential marker for infectivity. In fact, Will et al. (21) were able to infect chimpanzees with HBV DNA without DNA polymerase.

The effect of reducing the antigenicities of S and pre-S2 antigens by sodium hypochlorite, which is reported to be effective for HBV, was higher and more rapid at 0.1-0.5% than the effect of 80% ethanol. At 1%, HBV DNA reactive with the primers disappeared after 1-min treatment, confirming the effect of this disinfectant on HBV in vitro. We also confirmed the disappearance of HBV DNA by 10-min treatment with 0.25% sodium hypochlorite using a different method (Transcription-mediated amplification + Hybridization protection assay) (unpublished data). Bond et al. (22) reported loss of infectivity after 10-min treatment at 0.05% at room temperature in a chimpanzee experiment.

Prince et al. (23) showed that the enveloped virus, HBV, was more sensitive to chemical disinfectants than expected at the time the virus discovered. Our findings in this study suggest that one mechanism of loss of HBV infectivity by ethanol is blockage of the binding to hepatocytes caused by degeneration of the envelope.

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