—**Original**—

Effects of SSM (Specific Substance Maruyama) on HBe Antigen-Positive Chronic Hepatitis B

Clinical Efficacy and Modulation of Cytokines

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Abstract

Twenty-three patients with HBe antigen-positive chronic hepatitis B were treated with capitalite first letters Maruyama (SSM). HBe antigen turned negative in 15 patients. The levels of various cytokines in pre- and post-treatment frozen serum samples from six patients whose HBe antigen turned negative and from five whose HBe antigen did not were examined. Reduction of serum interleukin (IL) -10 level to below 20 pg/m*l* was observed after SSM treatment in four of the six patients whose HBe antigen turned negative. SSM was found to stimulate the production of interferon (IFN) -γ in peripheral blood cells from two healthy volunteers. This stimulatory effect was confirmed in 12 out of 24 healthy volunteers. SSM augmented the production of IFN-γ in eight out of 10 patients with chronic hepatitis B and nine of 10 with hepatitis C. These results demonstrate for the first time that SSM stimulates the production of IFN-γ in human peripheral blood cells and also suggest that treatment of HBe antigen-positive chronic hepatitis B patients with SSM leads to the clearance of HBe antigen and normalization of serum aspartate aminotransferase levels through inhibition of IL-10 and stimulation of IFN-γ (J Nippon Med Sch 2000; 67: 261—266)

Key words: SSM (special substance Maruyama), HBeAg positive chronic hepatitis B, cytokine, interleukin-10. interferon-γ

Introduction

Most cases of chronic hepatitis B are transmitted through mother-to-child infection. Later in its natural course, serum HBe antigen is cleared and serum aminotransferases normalize leading to alleviation of hepatitis. However, some patients suffer from persisting hepatitis and such patients are treated with interferon, corticosteroids, and more recently, with lamivudine.

In 1978, Brozosko *et al* reported that they treated 19 pediatric HBs antigen-positive chronic hepatitis patients with Bacillus Callete Guerin (BCG) and observed clearance of HBs antigen in seven patients[1]. Similarly, Bassedine *et al* found that treatment with BCG resulted in alleviation of inflammation and clearance of HB virus-associated antigens in three out of nine patients[2]. Special Substance Maruyama (SSM) is a substance separated and extracted from human *My-*

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Table 1 Response to SSM treatment

	12 mos of treatment	24 mos of treatment
HBeAg negative	8/23 (34.8%)	15/23 (65.2%)
anti-HBe positive	5/23 (21.7%)	7/23 (30.4%)
HBsAg negative	0	0
normal AST (< 35U/L)	9/23 (39.1%)	14/23 (60.9%)

HBeAg, hepatitis B e antigen ; anti-HBe, hepatitis B e antibody ; HBsAg, hepatitis B surface antigen ; AST, aspartate aminotransferase

cobacterium tuberculosis by Maruyama. Clinical trials on malignant tumors are currently underway[3]. We have treated HBe antigen-positive chronic hepatitis B patients with SSM and experienced cases where clearance of HBe antigen and normalization of aminotransferases were achieved. This observation was previously reported by Okumura et al[4] and Fujisaki et al[5].However, we did not have sufficient data to judge whether it was a real effect of SSM or a natural sequela of the disease. Therefore, to find clues to answer this question, we measured serum levels of various cytokines in frozen samples from patients with chronic hepatitis B before and after SSM therapy. Moreover, we studied the effects of SSM on the production of cytokines in normal individuals and patients with chronic hepatitis B or C.

Materials and Methods

(1) *SSM treatment and cytokine levels of patients with chronic hepatitis B*

Twenty-three patients with HBe antigen-positive chronic hepatitis B received treatment with SSM A solution containing 2 μg/m*l* of polysaccharide extracted from human *Mycobacterium tuberculosis* for a period of two years between October 1981 and December 1988. **Table 1** shows the numbers of patients who turned negative for HBe antigen and who had seroconversion or normal serum aspartate aminotransferase (AST) levels at 12 months from the start of and at the end of the treatment period. Serum levels of interferon (IFN)-γ, interleukin (IL)-2, IL-4, and IL-10 in the pre- and post-treatment frozen samples were determined by enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostic) in 11 of the 23 patients. For six patients in whom HBe antigen turned negative (effective group), sera taken before SSM therapy and when HBe antigen was cleared, and for five in whom HBe antigen persisted (ineffective group), those taken before and at two years from the start of therapy were used for the assay.

(2) *Cytokine production in the peripheral blood cells and RT-PCR*

Aliquots (1 m*l*) of heparinized blood samples from two healthy male volunteers, aged 32 and 43 years, respectively, were put into a 24-well plate within two hours and mixed with SSM A solution (0.1 m*l*). After being incubated 24 hours at 37℃, they were transferred to 1.5 m*l* microcentrifuge tubes and centrifuged at 12,000 rpm. Then the supernatants were stored at −80℃ until use. Levels of IL-1 α, IL-6, IL-8, IFN-γ, tumor necrosis factor (TNF)-α and granulocyte-colony stimulating factor (G-CSF) were determined twice in the independent fashion with an ELISA kit (Roche Diagnostic). Reverse transcriptase-polymerase chain reaction (RT-PCR) was carried out to study the transcripts of IFN-γ. Peripheral blood mononuclear cells (PBMC) were separated from heparinized blood samples by the Ficoll gradient method and transferred to a 12-well plate, They were then stimulated with 10% SSM in RPMI media containing 10% fetal calf serum for 4 hours at 37℃ in a CO_2 incubator. After stimulation, the cells were solubilized by adding 0.5 m*l* Isogen solution and stored at −80℃ until use. RT-PCR was carried out by the standard technique and the products were analyzed on 1.5 % agarose gel.

The stimulatory effect of SSM on the production of IFN-γ in normal subjects was similarly examined in 24 healthy volunteers (12 male, 12 female; mean age 33.6 years). IFN-γ production of the peripheral blood cells was also studied in 10 patients with chronic hepatitis B (7 male, 3 female; mean age 40.6 years) and 10 with

chronic hepatitis C (2 male, 8 female; mean age 61.7 years) before and after stimulation with SSM.

Results

1. Results of SSM therapy of HBe antigen-positive chronic hepatitis B patients

HBe antigen-negativity was defined by a cut off index of lower than 1.0. According to this criterion, 8 of 23 patients (34.8%) became HBe antigen-negative after one year and 15 patients (65.2%) after two years of treatment (**Table 1**). The percentage of patients who had so-called seroconversion, where the HBe antigen turns negative and the HBe antibody becomes detectable, was 21.7% after one year and 30.4% after two years. The HBs antigen did not become undetectable in any patient. Serum AST levels normalized in 14 of 15 patients (93.3%) whose HBe antigen turned negative. The changes in the AST values of these 15 patients in every three-month period from the start of SSM therapy are presented in **Fig. 1**. Transient elevation of AST between 3 and 9 months was observed in 9 patients (60%).

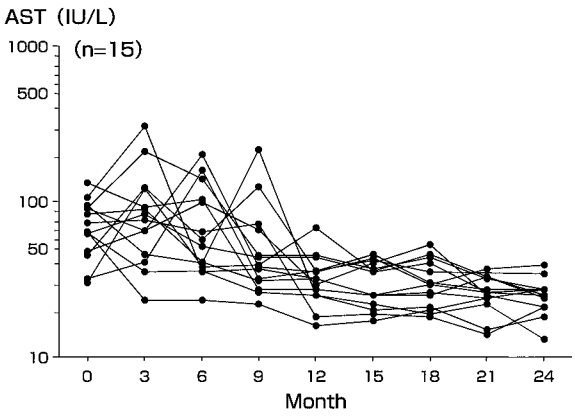

Fig. 1 Changes of AST levels during SSM treatment in patients whose HBe antigen turned negative. Changes in serum AST levels at the end of every three-month period in patients whose HBe antigen turned negative (effective group) are shown.

We defined patients whose HBe antigen turned negative with SSM therapy as the "effective group" and those whose HBe antigen persisted as the "ineffective group". There was no significant difference between the two group when SSM therapy was started, in age, sex, cut off index of HBe antigen, HBV DNA polymerase level, or histological findings (**Table 2**).

2. Changes in the cytokine levels before and after SSM therapy

The changes in cytokine levels in sera from the patients in the effective and ineffective groups before and after SSM therapy are shown in **Table 3**. Serum levels of IFN-γ, IL-2, and IL-4 were almost unchanged after SSM therapy in both groups. The IL-10 level was below 20 pg/m*l* before SSM treatment in two patients in the effective group and one in the ineffective group. Among patients with pretreatment IL-10 levels above 20 pg/m*l*, it decreased to below 20 pg/m*l* in all four patients in the effective group; however, no such decrease was observed in any of the four patients in the ineffective group.

3. Effects of SSM on the production of cytokines in normal subjects

Table 4 shows the production of cytokines in blood

Table 2 Comparison of basal characteristics betwen effective and ineffective groups

	Effective group	Ineffective group	p value
No. of patients	15	8	
Age (years) (mean ± SD)	35 ± 16	36 ± 9	NS
Sex (M/F)	9/6	4/4	NS
HBeAg (cut of index) (mean ± SD)	4.9 ± 2.1	5.4 ± 1.7	NS
HBV DNA polymerase (cpm/ml) (mean ± SD)	546 ± 525	1,319 ± 1,191	NS
AST (U/L) (mean ± SD)	72 ± 29	150 ± 96	< 0.05
Histological diagnosis			
chronic persistent hepatitis	3	2	NS
chronic aggressive hepatitis (moderate)	9	3	
chronic aggressive hepatitis (severe)	3	3	

NS : not statistically significant.

Table 3 Serum Levels of cytokines before and after SSM treatment in patients with HBe antigen-positive chronic hepatitis B

	patient	IFN-γ (pg/ml)		IL-2 (pg/ml)		IL-4 (pg/ml)		IL-10 (pg/ml)	
		pre	post	pre	post	pre	post	pre	post
Effective group	H.I	63	41	< 50	< 50	< 20	< 20	< 20	< 20
	K.O	47	149	< 50	< 50	< 20	< 20	< 20	< 20
	H.N	112	61	155	< 50	< 20	< 20	75	< 20
	Y.O	37	74	< 50	< 50	< 20	< 20	87	< 20
	M.Y	374	40	< 50	< 50	< 20	< 20	45	< 20
	Y.F	28	< 20	< 50	< 50	< 20	< 20	108	< 20
Ineffective group	K.U	1,122	191	< 50	< 50	21	< 20	254	94
	Y.H	59	144	< 50	< 50	< 20	< 20	< 20	< 20
	N.O	571	< 20	< 50	< 50	< 20	< 20	78	44
	T.T	56	269	< 50	< 50	< 20	< 20	74	56
	S.K	442	114	< 50	< 50	< 20	< 20	68	60

Table 4 Cytokines produced by SSM in two healthy subjects

Subject		IL-1α	IL-6	IL-8	IFN-γ	TNF-α	G-CSF
Subject-1	SSM	10 >	20 >	186	63	20 >	20 >
	Saline	10 >	20 >	149	20 >	20 >	20 >
Subject-2	SSM	10 >	20	134	89	41	20 >
	Saline	10 >	20 >	445	20 >	20 >	20 >

IL, interleukin ; IFN, interferon ; TNF, tumor necrosis factor ; G-CSF, granulocytecolony stimulating factor
SSM : SSM A solution 0.1ml. Values are expressed as pg/ml.

Table 5 IFN-γ before and after stimulation with SSM in patients with chronic hepatitis type B and C

Patient No	Type	IFN-γ (pg/ml)	
		Before	After
1	HBV	1.6	3.6
2	HBV	19.6	84.3
3	HBV	32.4	70.5
4	HBV	280.8	344.9
5	HBV	1 >	1 >
6	HBV	496	588.5
7	HBV	201.7	333.9
8	HBV	8.8	7.8
9	HBV	1.2	3.4
10	HBV	1 >	1.3
11	HCV	1 >	4.1
12	HCV	3,415.4	3,602.0
13	HCV	6.8	9.3
14	HCV	1 >	4.2
15	HCV	1 >	1 >
16	HCV	1 >	17.0
17	HCV	184.6	569.0
18	HCV	5.5	20.5
19	HCV	1 >	11.4
20	HCV	1 >	6.0

cells from two normal subjects stimulated by SSM. When the values obtained with saline were used as a control, significant increases in the production of IFN-γ in patient 1 and IL-6, IFN-γ, and TNF-α in patient 2, respectively, were observed. The production of IFN-γ was stimulated in both. While IL-8 levels were increased in both, they also increased with control saline. IFN-γ transcripts were detected after SSM stimulation in both (data not shown).

4. Stimulation of the production of IFN-γ by SSM in normal subjects

The stimulatory effect of SSM on the production of IFN-γ in normal subjects was further examined in 24 healthy volunteers and was observed in 12 of them. The levels of IFN-γ varied between 1.8 and 126 pg/ml (mean: 25.6 ± 33.1 pg/ml).

5. Production of IFN-γ after SSM stimulation in patients with chronic hepatitis B or C

The production of IFN-γ was already noted before stimulation with SSM in 8 of 10 patients with chronic

hepatitis B and in 4 of 10 with chronic hepatitis C. SSM stimulation augmented IFN-γ production in the peripheral blood cells of 8 patients with chronic hepatitis B and 9 with chronic hepatitis C (**Table 5**).

Discussion

Between 1981 and 1988, we treated 23 HBe antigen-positive chronic hepatitis B patients with SSM and, after a two-year treatment period, we found that HBe antigen turned negative in 15 patients (65.2%). Patients with chronic hepatitis B often experience clearance of HBe antigen during the natural course. However, such an event is reported to occur in only 15% of patients each year[6]. Accordingly, a rate as high as the 65.2% observed in this study is still high, even over a period of two years. In addition, transient elevation of AST levels was observed in nine of 15 patients between three and nine months from the start of the therapy, and AST levels ultimately normalized in 14 patients by the end of the two-year treatment period. It is well documented that transient hepatic injury manifested by elevation of aminotransferases frequently occurs when HBe antigen is cleared, and the hepatitis subsequently subsides[7]. Similar hepatic injury observed in the present study suggests that the immune response evoked by SSM is involved in the process. To confirm this observation, we studied pre- and post-SSM treatment changes in serum cytokine levels in frozen samples from 11 patients with chronic hepatitis B treated with SSM. Reduction of IL-10 levels to below 20 pg/m*l* following SSM therapy was seen only in the patients whose HBe antigen turned negative. In addition, cytokine production of peripheral blood cells was studied. We used unfractionated blood cells to minimaize the stimulation when PBMCs are fractionated and have a possible contamination of lipopolysaccharide in the medium which may also stimulate cytokine production. Other considerations were taken in the experimental procedures. Heparin was used as on anticoagulant, as EDTA and citrate inhibit production of cytokines. As the period between sampling and assay increases, there is a gradual decrease in cytokine production so cultures were started within two hours of sampling. Higher levels of cytokines were observed when stimulated at an ambient temperature than at 4℃. The concentration of lipopolysaccharide, blood dilution, and incubation period were those which gave the best production of cytokines in the preliminary experiments. Thus, peripheral blood cells from two healthy volunteers were stimulated by SSM under optimal experimental conditions. Production of IFN-γ was confirmed in both, and this stimulation was also observed in 12 of 24 (50%) healthy subjects, suggesting that there are two populations with regard to the response to SSM; responders, and nonresponders. There was no difference in age or sex between the responders and nonresponders.

Finally, we studied the effect of SSM on peripheral blood cells from patients with chronic hepatitis B or C. Eighty percent and 30% of patients with chronic hepatitis B and C, respectively, had elevated serum IFN-γ levels before stimulation. It is well recognized that IFN-γ levels are frequently elevated in patients with chronic hepatitis B[8].SSM augmented IFN-γ production in 80% of chronic hepatitis B patients and 90 % of chronic hepatitis C patients. These findings suggest that the clearance of HBe antigen, normalization of AST, and reduction of IL-10 observed in patients with chronic hepatitis B during SSM therapy are associated with the modulation of IFN-γ levels. Hayashi et al reported that IFN-γ is induced by SSM in mice[9]. To the best of our knowledge, however, no such study on humans has been published. This study proved for the first time that IFN-γ production is generated by SSM stimulation in patients with chronic hepatitis B or C.

Hepatic injury in chronic hepatitis B is not caused simply by infection of hepatitis B virus (HBV) to hepatocytes, but involves the immune response of cytotoxic T lymphocytes (CTL) specifically sensitized to HBV-related antigens expressed on HBV-infected hepatocytes[10].Thus, when a host immune system featuring CTL against HBV is activated, it attacks infected hepatocytes leading to hepatic injury. As HBe antigen is a target of CTL[11], hepatocytes infected with wild-type HBV which produces and secretes the HBe antigen will be destroyed by CTL and cleared. It should be mentioned that IFN-γ is reported to activate CTL[12].

Based on these findings, the results of this study lead to the following hypothesis: SSM treatment of pa-

tients with HBe antigen-positive chronic hepatitis B stimulates production of IFN-γ, which in turn activates CTL leading to clearance of HBV and alleviation of hepatitis. Moreover, as IL-10 secreted by Th 2 cells inhibits CTL activity which is modulated by Th 1 cells[13], SSM treatment might also exert an effect through reduction of serum IL-10 levels in patients in whom the HBe antigen turned negative.

In conclusion, this study confirmed for the first time that SSM stimulates the production of IFN-γ in human peripheral blood cells and suggested that SSM treatment of HBe antigen-positive chronic hepatitis B patients leads to clearance of HBe antigen and normalization of AST through reduction of IL-10 and production of IFN-γ.

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