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Studies of Hepatitis B Virus-Specific Transfer Factor Preparation

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Abstract To establish a new method for the preparation of hepatitis B virus-specific transfer factor preparation (HBV-STF), and hence some available data for comparative study of clinical application are documented. HBV-STF was prepared with human placental from HBsAg female patients positive. The physical and chemical quality and its immunological properties of HBV-STF preparation were tested. Also preliminary clinical trial was used, demonstrates a consistency in quality among each batch. Results from bacteria culture, safety and pyrogen tests and acute toxicity in mice all satisfied with the requirements of the pharmacopoeia, indicating that the preparations were safe. The maximum absorption of ultraviolet spectra was at 256 ± 2 nm, The $E_{260\text{nm}}/E_{280\text{nm}}$ is greater than 2.7, which is better than the internationally used criterion, i. e. $E_{260\text{nm}}/E_{280\text{nm}} \geq 1.8$. The preparations contained 17 kinds of amino acids. Effect of HBV-STF on activation of E receptors of human T lymphocytes shows the increasing rate ranging from 83.47% to 103.48%, HBV-STF can stimulate multiplication of T lymphocyte and inducing delayed allergy in mouse metatarsophalangeal skin by antigen specific skin test, indicating the preparations have strong immunological activities. Preliminary results from clinical trial also have obvious curative effect. The results showed that the HBV-STF preparation was a safe and high effective immuno-regulator for treating viral hepatitis B.

Key Words Hepatitis B virus-specific transfer factor (HBV-STF), Preparation, Physical and chemical quality, Immunological activities

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The transfer factor preparation (TF) is an immunological active mixture which is present inside white blood cells and dialysate. The specific transfer-factor preparation (STF) is a mixture which depends on its antigen for the biological activities^[1,2]. The nature of STF provides us with a new way for preparation. Our laboratory extracted HBV-STF from HBsAg positive human placenta, detected some of the properties of HBV-STF and conducted the preliminary clinical trial of the preparation. The results are reported here.

1 Materials and Major equipment

1.1 Materials

HBsAg positive human placenta were provided

by the Department of Obstetrics and Gynecology at Haizhu Women and Children hospital. Dialysis tubes with molecular weight cutting off 10 kD were from France. Hybrid mice (male and female, 17~28 g) were provided by department of animal of our college, New Zealand white rabbits (male and female, 2.5~3.4 kg) were provided by the Biological Institute of Guangdong.

1.2 Major equipment

MDF-292 ultra-low freezer (-85 °C, Sanyo, Japan); ZK 380 ultracentrifuge (Rerthold Hermle GbHoe Co., Germany); PCR thermal cycler (EPOCH Co., Tianjin); Amino acid analyzer (Walters, USA); 752 UV-spectrophotometer and 722 Visible-light spectrophotometer (The third factory for anal-

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ysis instruments of Shanghai).

2 Methods

2.1 Preparation of transfer factor

After fat and connective tissues were removed, HBsAg positive placenta were homogenized at high speed with equal amount of 0.9% NaCl. The homogenate were stored at -85°C overnight and thawed at 37°C . After several cycles of freezing and thawing, the supernatants were collected with ultracentrifugation and sterilized via dialysis and filtration. The bacteria culture, pyrogen test, animal toxicity, HBV-PCR and HBsAg test were performed to ensure the safety of the preparation. The preparation was packaged at 2 ml/Ampule.

2.2 Polypeptide contents mensuration

It was measured by Lowry method.

2.3 RNA content mensuration

It was measured by orcinol test.

2.4 HBV-PCR detection

PCR analysis were performed according to the manufacturer's instruction. The PCR products were electrophoresized in 2% agarose gel and compared with the positive control at 314 bp position.

2.5 Immunological activity assays

The immunological activity assays were formed with Ea-RFc method^[3]. Briefly, pancreatin (Difco, Detroit, MI, USA) was dissolved in Hank's solution (1%) and stored at -20°C . The control tube was added with 0.2 ml supernatant and tissue culture medium RPMI 1640 without TF. The experimental groups were added with 0.1 ml HBV-STF, P-TF and RPMI 1640 medium. The total volume in each tube was 1 ml. After incubated at 37°C for 18 hours, each tube was washed with 5 times of ice-cold Hank's solution twice. The pellets were resuspended in 0.2 ml RPMI 1640 for Ea-RFc assays^[4]. The results were calculated as following:

Ea-RFc% average accretion rate =

$$\frac{\text{experment group Ea-RFc\%} - \text{control group Ea-RFc\%}}{\text{control group Ea-RFc\%}} \times 100\%$$

2.6 Antigen specific skin test

Thirty healthy NIH mice of about 20 g each in weight (provided by the Experimental Animal Center of Zhongshan Medical University) were divided into 3 groups. The subcutaneous infection of HBV-STF 0.5 ml was given into the abdomen of each mouse in the tester group, once every other day 7 times altogether. A week after the last injection, the feet of the hind legs on both sides of themice were injected with 1:25 hepatitis B virus vaccine 0.5 ml (produced by Shenzhen Kangtai Biological Products company Ltd.) 72 hours after the injection, the hind legs on both sides of all the mice were cut off respectively. Pathological section was then done of the metatarsus skin taken from the hind legs after formaldehyde fixation, for the purpose of the observation of the infiltration of the lymphocyte in derma and subcutaneous tissues. The control group, with the substitutes of P-TF (produced by Guangdong Biological Products Institute) and physiological saline for HBV-STF, was employed for integration control.

3 Results and Discussion

3.1 HBV-STF quality control results

Tab 1 Detection results of HBV-STF

Item	941223	950120	950309
Aspect	Δ	Δ	Δ
pH	6.9	6.8	6.9
Ultraviolet spectra			
$E_{254\text{nm}}$	0.801	0.839	0.846
$E_{260\text{nm}}/E_{280\text{nm}}$	>2.70	>2.75	>2.90
Protein reaction	-	-	-
Polypeptide(mg/ml)	0.37	0.39	0.41
RNA(mg/ml)	0.19	0.20	0.22
HBsAg	-	-	-
HBV-PCR	-	-	-
Asepsis test	Δ	Δ	Δ
Safety test	Δ	Δ	Δ
Activity test(%)	Δ	Δ	Δ
Total evaluation	Δ	Δ	Δ

- Negative reaction, Δ Past master

Tab 1 demonstrates a consistency in quality a-

mong each batch. Results from bacteria culture, safety and pyrogen tests and acute toxicity in mice all satisfied the requirements of the pharmacopoeia, indicating that the preparations were safe. Since the preparations were extracted from HBsAg positive placenta, inactivation of HBV was critical. Wang *et al*^[6] incubated a similar preparation at 56 °C for 10 hours and treated it with 0.25% formalin. We found that although HBsAg was negative. The figure and Table 1 show the results of the random tests of three batches of preparations. The immunological activity of the preparation *in vitro* was also negative. We believe that TF is a heat-sensitive and will be denatured at 56 °C for just 45 min. The method reported here allowed us to remove HBV and even residual HBV-DNA at 4 °C as determined by PCR and HBsAg detection assays. More importantly, the methods were able to preserve the HBV-STF activity.

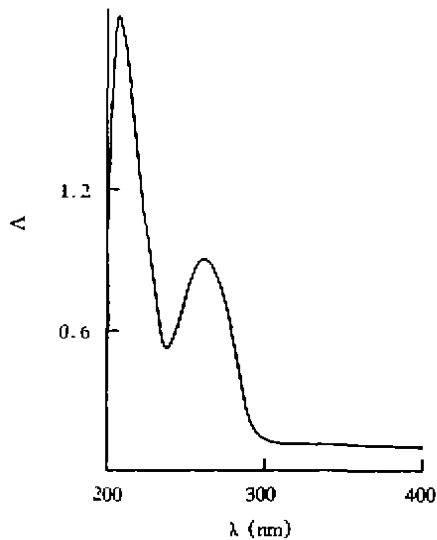


Fig 1 Ultraviolet spectra of HBV-STF

The figure 1 indicates that the preparation had two peaks appeared from 200 nm to 300 nm, which was similar to other TF. The maximum absorption was at 256 ± 2 nm. There was a parallel absorption from 254 ~ 260 nm with more occurred at 254 nm. This position was similar to that at which placental absorption occurred, suggesting that the preparation of HBV-STF may contain placenta STF^[6]. The $E_{260\text{nm}}/E_{280\text{nm}}$ is greater than 2.7, which is better

than the internationally used criterion, i. e. $E_{260\text{nm}}/E_{280\text{nm}} > 1.8$.

3.2 Contents of amino acids

Tab 2 illustrates the contents of amino acids. The preparations contained 17 kinds of amino acids.

Tab 2 Contents of amino acids in HBV-STF

Amino acid	Contents (mg/dL)	Amino acid	Contents (mg/dL)
Asp	1.867	Tyr	1.202
Glu	10.666	Val	10.678
Ser	1.799	Met	2.516
Gly	8.790	Cys	0.221
His	3.587	Ile	4.620
Arg	0.189	Leu	16.044
Thr	4.082	Phe	7.496
Ala	13.088	Lys	5.102
Pro	16.544	General contents	108.5

3.3 Effect of HBV-STF on activation of E receptors of human T lymphocytes

Table 3 shows the results from Ea-RFc assays. The second national meeting on TF adopted Ea-RFc or Et-RFc test as a standard method to determine the immunological activity of TF with a satisfactory value of greater than 20% increasing rate^[8]. We obtained the increasing rate ranging from 83.47% to 103.48%, indicating the preparations have strong immunological activities.

Tab 3 Effect of HBV-STF on activation of E receptors of human T lymphocytes

Group	N	Ea-RFc% ($\bar{x} \pm s$)	Incremental average value(%)
941223	5	21.1 ± 2.3	83.47**
950309	5	23.4 ± 3.6	103.48**
950401	5	22.3 ± 4.5	93.91**
P-TF	5	15.2 ± 2.8	32.26*
Control	5	11.5 ± 3.2	

* $P < 0.05$, ** $P < 0.01$, compared with control

3.4 Immunological specificity of HBV-STF

The mouse's skin was injected with HBV-STF, P-TF and physiological saline respectively for the delayed allergy. The infiltrate status of the lympholeukocyte in

shin was observed. The criteria for evaluation is 1 score for a little and scattered infiltrate lymphocyte, 2 score for more and asystematic infiltrate lymphocyte or focus concentrated infiltrate lymphocyte, 3 score for asystematic and focus concentrated infiltrate lymphocyte in the dermis of the mouse. The results were showed as table 4.

Tab 4 The results of pathological slice of treated metatarsophalangeal skin of mouse

Groups	Sample amounts	Integration of the records ($\bar{x} \pm s$)
HBV-STF	10	2.00 ± 0.67**
P-TF	10	1.60 ± 0.82*
Physiological saline (control sample)	10	1.30 ± 0.55

* $P < 0.05$, ** $P < 0.01$, compared with control

The result shows that HBV-STF and P-TF can stimulate multiplication of T lymphocyte and inducing delayed allergy in mouse metatarsophalangeal skin. But the effect of HBV-STF is more distinct than the effect of P-TF ($P < 0.01$ for HBV-STF). It manifests HBV-STF has the special cell immunological function of killing hepar B virus. That is HBV-STF possesses the immunological activity of depending on the antigen of hepar B virus.

3.5 Preliminary results from clinical trial

The preparations were used to treat 232 cases of chronic hepatitis and HBsAg carriers in the Red Cross Hospital of Shenzheng, of which, 78 patients were followed up. We found that 26.7% of patients became HBsAg negative, 58.3% HBeAg negative and 32.5% anti-HBc negative. After one therapeutical term (1 ampule/day, i. m. for 21 days), five patients with chronic hepatitis for 10 to 20 years became negative in "two vs half" 1, 3, 5 assays when they were tested 3 ~ 5 months later. The preparations were also used in conjunction with thymosin to treat 95 cases of hepatitis B patients. After one term of therapy, 31.1% of the patients became HBsAg negative, 46.3% HBeAg negative and 33.3% HBV-PCR

negative. In contrast, none of the patients became HBsAg negative, only 6.45 % of patients became HBeAg negative and 3.2% of the patients HBV-PCR negative in control groups ($P < 0.01$)^[9]. Moreover, the preparations were used in The People's Hospital of Shenzheng to treat 60 patients suffered from chronic hepatitis B. After three consecutive terms of therapy, the rates of patients who became negative with HBeAg, anti-HBc and HBV-PCR from positive were 64.6%, 52.3% and 60.0%, respectively. None of the patients has side or toxic reaction. Furthermore, the patients had increasingly good appetite and ameliorated insomnia after treatment. Therefore, we believe that the preparation is a safe and effective immuno-regulator for treatment which has a promising future. The biochemical nature and mechanisms of HBV-STF are still to be studied in addition to expand its clinical trial in the future.

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乙型肝炎病毒特异性转移因子的研究

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摘要 制备一种乙型肝炎病毒特异性转移因子制剂(HBV-STF),为临床应用提供有价值的实验依据。从人 HBsAg 阳性胎盘中制备了 HBV-STF,并对其理化性质、免疫学活性进行了检测和初步的临床试用。每批样品经无菌试验、热原质检查、动物安全性试验等均符合药典要求。本品最大紫外吸收光谱在 256 ± 2 nm 处, $E_{260\text{nm}}/E_{280\text{nm}}$ 比值大于 2.7。其水解氨基酸含 17 种。对人 T 淋巴细胞 E 受体的激活试验结果显示, HBV-STF 的 Ea-RFc 平均增高率在 83.47%~103.48% 之间,抗原特异性皮肤试验表明 HBV-STF 能刺激小鼠体内 T 淋巴细胞增殖,诱导小鼠跖趾部皮肤的迟发性变态反应。对 HBV-STF 的初步临床试用也取得明显效果,显示 HBV-STF 是一种可用于治疗乙肝的安全、有效的免疫调节剂。

关键词 乙型肝炎病毒特异性转移因子;制备;理化性质;免疫学活性

乙型肝炎, 免疫调节剂, 转移因子

《药物生物技术》论文在国内外著名文摘性刊物上的摘引情况

《药物生物技术》自 1994 年创刊以来,得到了广大作者和读者的关怀和支持。国内许多大学、研究所及医药界各单位的专家来稿(其中很多是国家及地方级重点科研项目)业务造诣很深,论文水平很高,对提高刊物的质量起了保证作用。这些论文大都收载到国内外的权威性文摘中。今将本刊各期论文在国内外两种文摘性期刊中被利用情况列入下表。

表 1 《药物生物技术》的文摘利用率

年 期	主要论文 篇 数	被摘篇数		被摘篇数		
		中国药学文摘	CA	中国药学文摘	CA	
1994	1	13	9	4	69	31
1994	2	15	10	4	67	27
1995	1	16	12	7	75	44
1995	2	15	11	7	73	47
1995	3	12	10	4	83	33
1995	4	17	10	13	59	76
1996	1	14	13	10	93	71
1996	2	16	14	13	88	81
1996	3	14	12	12	86	86
1996	4	14	14	14	100	100
1997	1	16	12	15	75	94
1997	2	14	13	14	93	100
1997	3	13	12	12	92	92
1997	4	15	13	13	87	87
1998	1	15	12	15	80	100
1998	2	15	14	13	93	87
1998	3	15	15	12	100	80
1998	4	16	11	10	69	63
1999	1	14	11	11	79	79
1999	2	14	14	待查	100	
1999	3	15	15	待查	100	

以后在本刊“信息反馈”中将陆续把各期文章在 CA 和《中国药学文摘》中被摘录的编号刊出,以供作者参考,并欢迎作者索取文摘。

《药物生物技术》编辑部