Brief Report

Preparation and Determination of Immunological Activities of Anti-HBV Egg Yolk Extraction

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To prepare an effective immune preparation to treat hepatitis B, hens were immunized with hepatitis B vaccines, and then anti-HBV egg yolk extraction (anti-HBV EYE) was refined from egg yolk by a dialyzable method. Its chemical characteristics were identified by ultraviolet spectrum, HPLC, Lowry analysis and pharmacopocia-raleted methods. The specific immunological activity was examined by leukocyte adherence inhibition (LAI) *in vitro* and delayed type hypersensitivity (DTH) *in vivo*. Anti-HBV EYE was a small dialyzable substance with molecular weight less than 12 kD containing 18 kinds of amino acids. The preparation could obviously inhibit LAI and DTH which was similar to hepatitis B virus-specific transfer factor of pig spleen. However, there were no similar effects observed in the nonspecific transfer factor (NTF) group, control egg yolk extraction (CEYE) group and hepatitis A virus (HAV) group. The results suggested that anti-HBV EYE contained hepatitis B virus-specific transfer factor (STF) and had the antigen-specific cell immune activity similar to PS_{HBV}-TF. The STF obtained from egg yolk of the hens immunized with specific antigen, might be a potential candidate for immunoregulation in hepatitis B prevention and treatment. *Cellular & Molecular Immunology*. 2006;3(1):67-71.

Key Words: egg yolk extraction, hepatitis B virus, specific transfer factor, immunological activity

Introduction

Transfer factor (TF) is a kind of lymphokines. It could transform unsensitized T lymphocyte into sensitized T lymphocyte. As an immunoregulation factor, TF has been widely used for treatment of some diseases (1, 2). Both nonspecific transfer factor (NTF) and specific transfer factor (STF) were prepared traditionally from human and animal leukocytes (3, 4). In addition, it has been recently reported that TF was also found in the immunized egg yolk (5, 6). However, it is still unclear whether the chemical characteristics and the immunologic activities of the egg yolk-related TF are similar to that existing in leukocyte.

In this study, hens were immunized with hepatitis B vaccines. The anti-HBV egg yolk extract (anti-HBV EYE) was refined from the egg yolk by Lawrence dialyzable method, and then its chemical characteristics and immunologic activities were assayed in comparison with the STF prepared from leukocytes so as to supply a possible method for clinical treatment of hepatitis B.

Materials and Methods

Animals and reagents

Eighteen-week-old hens were supplied by the Center of Domestic Fowl in Nanchang. KM mice, female, 18-20 g, were bred and maintained at the Animal Center, Jiangxi Medical College.

HBV vaccine was purchased from Biological Products Institute (Shanghai). The hepatitis B specific transfer factor of pig spleen (PS_{HBV}-TF) and HAV vaccine were purchased from Changchun Biotechnical Pharmaceutical Company. The pig spleen nonspecific transfer factors were products of the Biotechnical Company (Nanjing). High-performance liquid chromatograghy (HPLC) and UV-120 spectrophoto- meter and visible light spectrophotometer (SHIMADZU) were also used.

Animal immunization

Hens were injected subcutaneously with hepatitis B vaccines mixed with Freud's adjuvant four times at two-week interval at dose 10 μg per hen. After immunization, the sera were collected for detecting anti-HBs every two weeks (7).

The preparation of anti-HBV EYE

The egg yolks were isolated sterilely from the hens whose serum HBs Ab titre were above 1:80, and then homogenized

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at high speed. All fractions were put into 12 kD-cutoff dialysis tubing and dialyzed for 36 h with equal amount of deionized water at 4°C, then filtrated sterilely and stored at -40°C. The bacteria culture, pyrogen, animal toxicity and HBsAg test were performed to ensure the safety of the preparation. The control of egg yolk extraction (CEYE) derived from the material of un-immunized yolk was obtained by the same method as described above.

Analysis of general physio-chemical properties
Refering to formula for producing and determination of freeze-dried human TF in China biological pharmaceutical formula (8), the properties of anti-HBV EYE, including color, transparence, pH, polypeptides, ribose, protein reaction, hypersensitivity and pyrogenic test, were determined.

Analysis of ultraviolet spectrum and animo acids
The absorption peaks of the preparation scanned in full-wavelengh with ultraviolet spectrophotometer and the ratio of A260/A280 were recorded. HPLC was used to analyze animal acid of preparation.

Determination of immune activity
The nonspecific immune activity was analyzed by calculating thymus-spleen index of each mouse.

The specific immune activity was analyzed by delayed-type hypersensitivity (DTH) reaction test (9). Fifty KM mice were divided into five groups, *i.p.*, injected once a day for seven times. One group was treated with NS only as the control, while other four groups were treated with anti-HBV EYE, PS_{HBV}-TF, NTF and CEYE, respectively. A week after the last injection, the right hind paw skin of the mice was injected with 0.3 μg/30 μl hepatitis B virus vaccine and the left with 30 μl NS respectively; 24 h after injection, the thickness and width of hind footpad of a mouse were measured with micrometer. Perimeter of mice's hind footpad was expressed as follows: Perimeter = (thickness of hind footpad + width of hind footpad) × 2.

The specific immune activity was also analyzed by leukocyte adherence inhibition (LAI) (10). Mouse spleens were picked out and put on a stainless 200-mesh screen in dish, then 1 ml Hanks solution was added to the dish and the spleen was pounded into pieces. The cells were washed three times by centrifugation at 1,000 rpm for 10 min, then resuspended in RPMI 1640 containing 10% fetal bovine serum, adjusted to 2×10^6 cells/ml, and 1 ml cell suspension was mixed with 1 ml test substances (see results) for 1 h at 37°C and washed once. Each tube was added to 0.8 ml RPMI 1640 and 0.2 ml HBV vaccine (30 μg/ml) or HAV vaccine (as antigen control), then the materials were transferred from test tubes to 24-well plates. The plates containing the cell suspensions and test substances were kept in a 5% CO_2 incubator at 37°C for 1 h. After the incubation period, the plates were gently shaked three times, and supernatants were transferred to test tube. Under the microscope, unadherence cells in the test tubes were counted and the percentage of LAI is calculated as follows: LAI = (unadherence cells in test tubes - unadherence cells in NS control tubes) / (unadherence cells in NS control tubes).

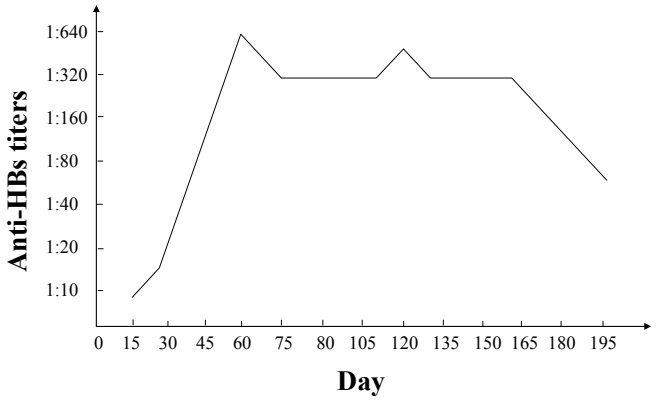

Figure 1. HBsAb titers in serum of hen vaccinated with hepatitis B vaccines. Hens were injected subcutaneously with recombinant hepatitis B vaccines with subsidiary adjuvant totally four times and the injection dose of each hen was 10 μg. Anti-HBs in serum was detected by ELISA.

Statistical analysis
Data analysis was performed using unpaired student's *t*-test. Differences of $p < 0.05$ were considered to be significantly different from control.

Results

Humoral immune responses induced in hen by HBV antigens
The anti-HBs antibody test result was negative in serum of hen before being immunized with HBV antigens and positive conversion by immunization for two weeks. The anti-HBs peak value reached 1:640 on day 60 and 120 after vaccination and anti-HBs titers still maintained at 1:80 on day 195 (Figure 1).

General physio-chemical properties of the anti-HBV EYE
The preparation is a small dialyzable substance with molecular weight less than 12 kD and pH value of 6.9 ± 0.5. The extraction has polypeptides content 1.20 ± 0.47 mg/ml and ribose up to 152.94 ± 2.83 μg/ml, containing 18 kinds of amino acids with a concentration of 1268.4 μg/ml (Table 1). Furthermore, no protein reactions were detected.

Analysis of ultraviolet spectrum of the anti-HBV EYE
Scanned in the range between 200 nm and 300 nm with ultraviolet spectrophotometer, the anti-HBV EYE had an absorption peak at 270 nm, and the A260/A280 was 1.2. Comparatively, the PS_{HBV}-TF had an absorption peak at 254 nm, and the A260/A280 was more than 1.8 (Figure 2).

Effect of the anti-HBV EYE on DTH in mice
The results of hindpaw swelling volume in mice showed that, in the group injected with anti-HBV EYE, hindpaw swelling volume of the side attacked with HBV antigens was higher

Table 1. Contents of amino acids in Anti-HBV EYE

Amino Acids	Contents（μg/ml）
Asp	118.6
Glu	252.5
Ser	77.9
Arg	91.3
Gly	47.9
Thr	40.8
Pro	65.4
Ala	46.7
Val	64.9
Met	25.1
ILe	54.3
Leu	99.4
Phe	59.6
His	25.8
Lys	91.3
Tyr	80.1
Cys	12.4
Trp	14.4
Total	1268.4

than that of the NS control side (1.75 ± 0.12 and 1.56 ± 0.09, $p < 0.01$) which was similar to the group injected with PS_{HBV}-TF (1.70 ± 0.12 and 1.54 ± 0.13, $p < 0.01$). However, in other groups, there were no obvious differences between their attacked sides and their relevant NS control sides ($p > 0.05$), which suggested that the anti-HBV EYE, similar to PS_{HBV}-TF, could also induce DTH in mice (Table 2).

Effect of anti-HBV EYE on LAI

The results showed that the values of LAI of the groups containing anti-HBV EYE and HBsAg were 25.81 ± 3.94%, which was similar to that of the group containing PS_{HBV}-TF and HBsAg (27.95 ± 4.01%, $p > 0.05$) but significantly higher than that of the group containing CEYE and HBsAg (12.32 ± 1.13%, $p < 0.01$), the group containing NTF and HBsAg (15.24 ± 0.89%, $p < 0.01$) and the group containing anti-HBV EYE and HAAg (6.25 ± 1.50%, $p < 0.01$). It suggested that both the anti-HBV EYE and the PS_{HBV}-TF could inhibit leukocyte adherence of mice *in vitro*, and the effect of LAI was dependent on specific antigen, but not other groups (Table 3).

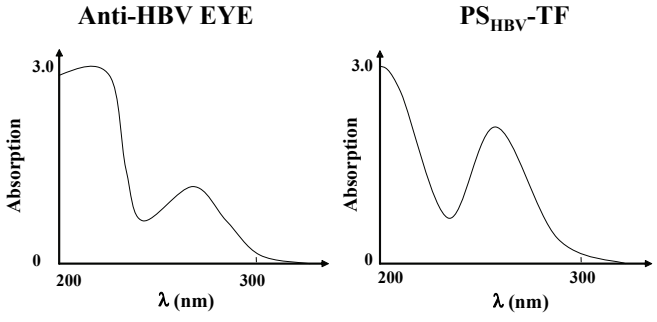

Figure 2. Ultraviolet spectra of egg yolk extraction against hepatitis B virus and PS_{HBV}-TF. The egg yolk extraction was scanned in the range between 200 nm and 300 nm with ultraviolet spectrophotometer.

Table 2. The results of hindpaw swelling volume in mice

Groups	Hindpaw swelling volume (cm)		*p*
	Control	Attack	
Control	1.55 ± 0.06	1.59 ± 0.11	> 0.05
Anti-HBV EYE	1.56 ± 0.09	1.75 ± 0.12	< 0.01
CEYE	1.53 ± 0.11	1.59 ± 0.14	> 0.05
PS_{HBV}-TF	1.54 ± 0.13	1.70 ± 0.12	< 0.01
NTF	1.58 ± 0.07	1.63 ± 0.08	> 0.05

The influence of anti-HBV EYE on thymus-spleen index of mice

The thymus-spleen index in mice was detected, and the results showed that it was increased significantly in four groups compared with the NS group ($p < 0.01$, $p < 0.05$), but there was no significant difference between the anti-HBV EYE and NTF ($p > 0.05$) (Table 4).

Biological detection

It acted in accordance with Chinese pharmacopeia in sterility test, safety test, hypersensitivity test and pyrogenic test.

Discussion

Since Lawrence described dialyzable leukocyte extract (DLE) containing TF in 1949, scientists have done a lot of researches on its theory and appliance. It has been shown that the extract contains at least 200 different moieties and only one of them is TF. It has been widely used in some diseases, and was effective (11, 12). Data from recent trials suggested

Table 3. The LAI effect of Anti-HBV EYE (n = 8)

Groups	LAI Index %
Anti-HBV EYE + HBsAg	25.81 ± 3.94*
CEYE + HBsAg	12.32 ± 1.13
PS_{HBV}-TF + HBsAg	27.95 ± 4.01
NTF + HbsAg	15.24 ± 0.89
Anti-HBV EYE + HAAg	6.25 ± 1.50
N.S + HBsAg	14.05 ± 4.75

*$p > 0.05$ *vs* PS_{HBV}-TF + HBsAg group, $p < 0.01$ *vs* other groups.

Table 4. The results of index in spleen and thymus

Groups	Thymus (mg)	Spleen (mg)
NS (Control)	2.377 ± 0.1765	2.840 ± 0. 3243
Anti-HBV EYE	3.543 ± 0.2713*$^{\Delta}$	3.470 ± 0.1784*$^{\Delta}$
CEYE	3.413 ± 0.1894*	3.489 ± 0. 3130*
PS_{HBV}-TF	3.629 ± 0.3171*	3.275 ± 0.2932*
NTF	3.903 ± 0.2297**	3.808 ± 0. 3142**

*$p < 0.05$, ** $p < 0.01$ *vs* NS control group, $^{\Delta}p > 0.05$ *vs* NTF group

that STF was generally more efficacious than NTF (13-15). Traditionally, both NTF and STF were prepared from human as well as animals such as pigs and bovines, because the TF exists in leukocytes. NTF can be made by traditional methods at much lower cost and in much less time than it required for the production of STF, because STF generally was prepared by immunizing huge animals such as pig, cattle with a large quantity of HBV antigens, but obtained only in spleen. Recently, studies have shown that hen was immunized successfully with antigens, and antibodies transported to its yolk through blood circulation, and a large amount of antibodies could be obtained from the yolk (16). This means that some cell-mediated immunity material may appear in the yolk, too. Deyuan Chen et al. (5, 6) proved the deduction, which suggested that the immune yolk contained substances which can induce cell-mediated immunity.

In this study, we found that hens were immunized with Hepatitis B vaccines, and then the egg yolk extraction was refined from egg yolk by a dialyzable method. This material, as mentioned above, was termed as anti-HBV EYE by us. The anti-HBV EYE was a small dialyzable substance with molecular weight less than 12 kD and contained 18 kinds of amino acids (total was 1,268.4 μg/ml). The poly-peptides content was 1.2 mg/ml and ribose was 152.94 ± 2.83 μg/ml. The extraction could obviously inhibit the adherence of leukocytes ($p < 0.01$) *in vitro* and induce significant delayed type hypersensitivity in mice footpad skin *in vivo* ($p < 0.01$), which was similar to hepatitis B virus specific transfer factor prepared from leukocyte of pig spleen ($p > 0.05$). The preparation was also found to be able to increase thymus-spleen index and stimulate proliferation of lymphocytes in mice. The data demonstrated that it possessed not only specific immune activity, but also nonspecific immune activity which was similar to NTF. In addition, other important parameters for identification were ultraviolet spectra and A260/A280 ration. The maximum absorption was at 270 ± 2 nm and A260/A280 was lower than 1.8, which didn't accord with the fact that the peak of spectrum was at 254 nm and A260/A280 was greater than 1.8 from TF of leukocytes. The reason may be related to the difference of materials (17). It was assumed that results should have been more satisfied if the preparation had been refined by ultrafilter method. Thus, abstracting methods and biochemical properties in anti-HBV EYE shall be studied further.

We concluded that the extractions from immune hen yolk contained some active materials which initiated cell-mediated immunity. One of the active materials is anti-HBV STF. Furthermore, the hen is relatively small and could be fed and managed easily. Once immunized successfully by a relatively small quantity of antigens, a hen can lay eggs continuously which contain STF in the yolk. It is easier, cheaper and comparatively securer to gain STF from yolk than to gain it from huge animals immunized with larger quantities of antigens. Thus, it is thought that anti-HBV EYE can be developed as a potential immunoregulation for preventing and treating hepatitis B.

Acknowledgement

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