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# ISOLATION AND PURIFICATION OF HSV-1 SPECIFIC TRANSFER FACTOR PRODUCED BY HSV-1 IMMUNIZED GOAT LEUKOCYTE DIALYSATE

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*Summary.* - A herpes simplex virus type 1 (HSV-1) - specific transfer factor (TF), was separated and purified from the leukocyte dialysate of goats immunized with HSV-1 using affinity chromatography on antigen-sorbent and reversed phase high performance liquid chromatography (RP-HPLC). The antigen-specific activities of the starting dialysate and the isolated TF component (s) were examined by $^{51}$Cr-labelled leukocyte adherence inhibition ($^{51}$Cr LAI) assay. The analytical hydrophobic interaction HPLC (HI-HPLC) and isoelectric focusing (IEF) techniques were employed to evaluate the purity and the isoelectric point (PI) of isolated TF component(s). The experiments provided a two-step procedure for purifying the TF material from the starting dialysate. It seems that the purified active TF component (PTFC) was specific for HSV-1. The specific PTFC activity was increased 10,000-fold as compared with the activity of the dialysate. The active moiety appeared as a single band in the IEF gel as demonstrated by silver staining; it was hydrophilic and its PI was pH 4.48.

*Key words: transfer factor; herpes simplex virus type 1; $^{51}$Cr-labelled leukocyte adherence inhibition assay; affinity chromatography; RP-HPLC*

## *Introduction*

Transfer factor (TF) can apparently transfer cell-mediated antigen specific immune responses from a highly sensitive donor to a nonsensitive recipient (Kirkpatrick *et al.*, 1985*a*). At present, crude TF preparations have been used for treatment of some viral diseases and cancers associated with a lower state of cell-mediated immunity (Fudenberg, 1988). However, TF represents only a minor fraction among many substances present in dialyzable leukocyte extract, so it is very difficult to separate and purify the active fraction from the total dialysate. Up to now, no homogeneous component responsible for

transfer of immunological activity has been obtained, and the biochemical characterization of TF has remained unclear so far.

A major concern in TF research has been the separation and purification of antigen-specific TF. The most conventional isolation techniques applied during the past years included size exclusion chromatography (Wilson *et al.*, 1978), precipitation and extraction of organics (Klesius and Fudenberg, 1977), ion exchange chromatography (Foster *et al.*, 1979) and thin layer chromatography (Wilson *et al.*, 1981). However, these methods were relatively insensitive, of low resolution power and sample-consuming so that TF could not be well separated. Development of RP-HPLC technique has offered a new means of excellent resolution and high sensitivity. As a result some partial success on TF purification has been achieved (Burger *et al.*, 1979; Paddock *et al.*, 1979). Subsequent finding of a specific interaction between TF and antigen (Borkowsky and Lawrence, 1981; Burger *et al.*, 1983) has further provided a new possibility by utilizing the property of TF binding to antigen (Kirkpatrick *et al.*, 1985*b*). Using this method, we successfully isolated the HSV-1 specific TF components from spleen cells of HSV-1 immunized mice. Meanwhile, a modified $^{51}$Cr-LAI test was used as a reproducible *in vitro* assay for measuring the antigen-specific activity of TF components (Huang *et al.*, 1987). The results showed that non-immunized mice which were injected by TF components exhibiting by antigen-dependent effect in LAI elicited a cutaneous delayed type hypersensitivity (DTH) to HSV-1 and protected them from challenge with a lethal dose of HSV-1.

However, homogenous TF active material has not yet been obtained either by RP-HPLC or affinity separation alone. Also the amount of starting TF sample from mice was limited so that it was difficult to purify the TF, to determine its biochemical properties and apply the purified TF for treatment. Here we report a two-step purification procedure including affinity chromatography and RP-HPLC for isolation and purification of specific TF active component from the HSV-1 immunized goat dialyzable leukocyte extract.

## *Materials and Methods*

*Preparation of the dialysate containing TF.* Male hybrid goats aged 1 were used. All animals were raised in our animals care facility. The goats were given the living HSV-1 strain SM44 by intradermal inoculation. The virus was grown in BHK cells in medium 199. The HSV-1 immunized goats exhibited marked DTH response to HSV-1 in skin test 4 weeks after immunization. The dialysate (TF-HSV-1) was prepared from spleen and lymph node cells by conventional homogenization and dialysis technique. Briefly, the splenic and lymph node cells were disrupted and the material was then placed in dialysis bags with a 10 000 molecular weight (M. W.) exclusion size and dialyzed against sterile distilled water at 4 °C for 48 hrs. The dialyzable materials were concentrated by lyophylization and reconstituted in 0.9 % NaCl solution (NS) to obtain absorbance 8.0 at 260 nm (1 unit of TF) per 1 ml. After filtration through 0.22 $\mu$m Millipore filters, the dialysate was stored at -20 °C. The control dialysate was prepared from spleen and lymph node cells of unsensitized goats (TFn) in the same manner.

*Assay for TF activity.* $^{51}$Cr-labelled leukocyte adherence inhibition ($^{51}$Cr-LAI) assay has been described elsewhere (Huang *et al.*, 1987).

*Affinity chromatography.* Batchwise affinity separation procedure of TF-HSV-1 on HSV-1 antigen-sorbent was made as described (Huang *et al.*, 1987).

*Reversed phase high performance liquid chromatography.* RP-HPLC was performed at room temperature. A Waters associate liquid chromatograph system with two Model 6000 A solvent delivery pumps, a Model 440 ultraviolet detector and a Model 660 system controller were used throughout separation. The lyophylized samples to be fractionated were dissolved in 0.1 % HPLC-grade trifluoroacetic acid (TFA) (Merck-Schuchardt, F.R.G.) and filtered through 0.45 $\mu$m Millipore filter. A 0.39 × 30 cm uBondapark C18 column (Millipore-Waters Associates) was employed. Solvents for RP-HPLC were 0.1 % TFA in HPLC-grade 90 % methanol (China) as the organic phase and 0,1 % TFA in HPLC - grade water as aqueous phase. Measurements were made at 280 nm in the sensitivity range of 0.05 absorption units full scale. The column was run at a flow rate of 0.7 ml/min. Fractions were collected following the appearance of each peak and dried in a speed Vac concentrator.

## *Results and Discussion*

### *Affinity separation of HSV-1 specific transfer factor*

On the basis of a specific interaction between TF-HSV-1 and HSV-1, affinity chromatography on the HSV-1 antigen-sorbent was used as first step for isolating TF active material from the starting dialysate. TF-HSV-1 could bind to HSV-1 antigen-sorbent, but it did not bind to the control sorbent lacking HSV-1 antigen, neither the TFn produced from nonimmunized donor goat could bind to HSV-1 antigen-sorbent (Table 1) indicating that TF-HSV-1 preparation contained the specific HSV-1 binding components. These TF-HSV-1 specific components (TFC) were eluted from the antigen-sorbent by changing the elution temperature and ionic strength of the eluate.

To determine the effect of affinity chromatography on TF-HSV-1, the activity and purity of TF components were examined in $^{51}$Cr-LAI assay and by analytic hydrophobic interaction HPLC technique. Tables 2 and 3 show the antigen-specific activity of TF-HSV-1 and TF components in $^{51}$Cr-LAI assay, respectively. The $^{51}$Cr-labelled leukocyte adherence inhibition indices (LAII)

**Table 1. Specific absorption of TF-HSV-1 to HSV-1 sorbent**

| Reaction system | Elution rate | Absorption rate | N |
|---|---|---|---|
| TF-HSV-1 + HSV-1 sorbent | 61.9 % ± 4.9 | 38.1 % ± 4.9 | 7 |
| TF-HSV-1 + Control sorbent | 98.7 % ± 1.3 | 1.3 % ± 1.3 | 2 |
| TFn + HSV-1 sorbent | 98.0 % ± 2.0 | 2.0 % ± 2.0 | 2 |

**Table 2. The antigen-specific LAI effect of TF-HSV-1**

| Groups | HSV-1 | TF-HSV-1 +MV[a)] | TF-HSV-1 +BHK | TF-HSV-1 | TF-HSV-1 +HSV-1 | TFn +HSV-1 |
|---|---|---|---|---|---|---|
| 1 | -4.9* | -5.5 | -7.4 | -4.5 | 16.0 | -19.7 |
| 2 | -0.4 | -9.8 | -4.9 | 5.1 | 24.1 | -1.9 |
| 3 | -3.9 | -0.6 | 1.0 | -2.7 | 13.0 | 0.2 |
| 4 | -16.7 | -15.3 | 3.2 | -9.8 | 14.6 | -18.6 |
| 5 | -5.1 | -0.4 | -5.4 | -11.0 | 14.6 | -9.0 |
| 6 | -0.9 | -0.9 | 1.5 | -3.6 | 17.9 | 1.6 |
| Mean | -5.3 | -5.4 | -2.0 | -4.4 | 16.7 | -7.9 |
| SE | 2.4 | 2.5 | 1.8 | 2.4 | 1.6 | 3.9 |
| P value[b)] | <0.01 | <0.01 | <0.01 | <0.01 | | <0.01 |

a) MV — Measles virus
b) Compared with TF-HSV-1 + HSV-1 group; the difference between the rest groups was not significant ($P>0.05$) using two way variance analysis
* LAII per cent

were used to demonstrate the TF specific activity *in vitro*. When leukocytes obtained from nonimmunized mice were incubated with the TF preparations, they became responsive to HSV-1 (the LAII obviously increased), but not to control antigen from normal BHK cells and to measles virus antigen. Thus,

**Table 3. The antigen-specific LAI effect of TFC**

| Groups | TFC+BHK | TFC+HSV-1 |
|---|---|---|
| 1 | 2.8* | 24.2 |
| 2 | -2.9 | 16.6 |
| 3 | -8.5 | 18.5 |
| 4 | 7.2 | 21.6 |
| 5 | -10.7 | 16.6 |
| 6 | -8.8 | 18.8 |
| Mean ± SE | -3.5 ± 2.9 | 19.4 ± 1.2 |
| P value[a)] | <0.01 | |

a) By the analysis of variance with two way
* LAII per cent

both the crude TF-HSV-1 and the isolated TF components exhibited HSV-1 specific activity. Since the concentration of TF components producing LAI effect ($2\times10^{-7}$ U/ml) was 1/5,000 of TF-HSV-1 ($10^{-3}$ U/ml) producing the same affect, it seemed that the activity of TF components had increased by 5,000-fold as compared with the activity contained in crude TF-HSV-1.

Analysis of hydrophobic interaction HPLC on TF components was performed on a 0.6 ×15 cm HIC-5J6 column (China) utilizing a Shimadzu LC-6A high performance liquid chromatograph equipped with two solvent delivery pumps and variable UV detector (Fig. 1). (Solvent system buffer A: 3 mol/l $(NH_4)_2SO_4$ and 10 mmol/l $KH_2PO_4$; buffer B: 10 mmol/l $KH_2PO_4$ was used for the separation). Only two UV absorbence peaks (at 220 nm) were eluted by a descendent salt gradient at a flow rate of 0.6 ml/min indicating that the partially purified TF active components was effectively isolated by affinity chromatography.

The above mentioned experiments showed that affinity chromatography used here was a mild and specific means for isolation the low concentration sample of antigen-specific TF from the crude dialysate. However, not all impurities could be removed by such one-step isolation.

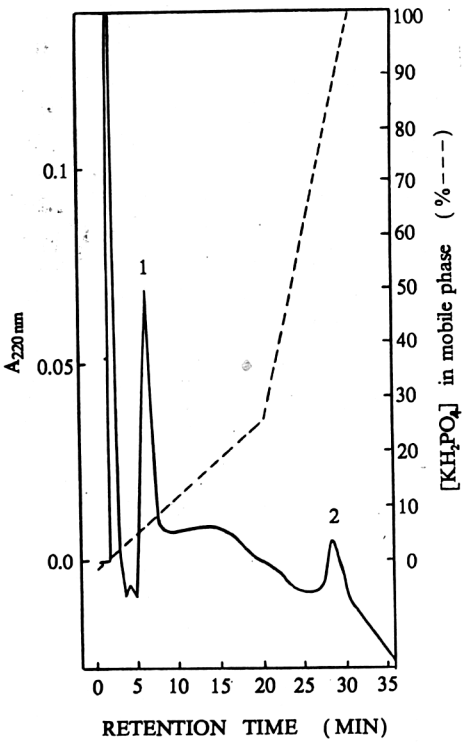


**Fig. 1**
Hydrophobic interaction HPLC of TFC on a HIC-5J6 0.6 × 15 cm column
The sample was eluted at 0.6 ml/min with a linear gradient, composition: $(NH_4)_2SO_4/KH_2PO_4$ 3 mmol/l/10 mmol/l. The gradient is shown by dotted line.

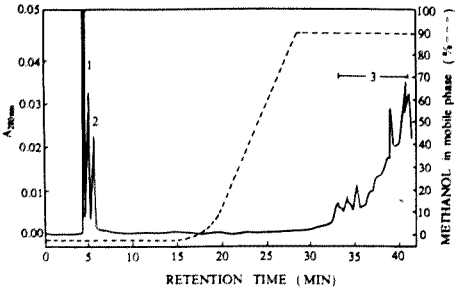


**Fig. 2**
Reversed-phase HPLC of TFC on a uBondapark 0.39 × 30 cm C18 column The sample was eluted at 0.7 ml/min with a linear gradient, composition: Methanol/Water/TFA 0/99.9/0.1 to 90/9.9/0.1 (v:v:v). The gradient is shown by dotted line.

*Reversed phase HPLC purification of HSV-1 specific TF components*

TF components concentrated by affinity chromatography were then purified by RP-HPLC. Three fractions of TF preparation were eluted from the C18 column (Fig. 2); which were collected, and their TF activity was evaluated by $^{51}$Cr-LAI assay. As shown in Table 4, TF specific activity was found only in fraction 2, but not in other fractions. Subsequently, the activity of the fraction 2 was compared with that of crude TF-HSV-1 (Table 5), showing that the final purified TF component was enriched 10,000-fold. Since fraction 2 was eluted from the RP-HPLC column within 6 min its hydrophilicity was also demonstrated.

The purity and isoelectric point (PI) of fraction 2 with TF specific acitivity were further determined by isoelectric focusing (IEF). The sample was analyzed on a thin layer of polyacrylamide gel with pH range of 3.5 - 5.5. When focusing was completed, the pH gradient of the gel was determined by the

**Table 4. Antigenic specific LAI effect produced by the fraction obtained from RP-HPLC of TFC**

| Fractions | Antigen | LAII %[a)] |
|---|---|---|
| Fraction 1 | HSV-1 | 1.3 ± 1.4 |
| Fraction 2 | HSV-1 | 17.9 ± 1.4 |
| Fraction 3 | HSV-1 | 1.9 ± 0.7 |
| None | HSV-1 | 3.3 ± 1.6 |

[a)] Values shown are Means ± SE from 4 independent experiments by two way analysis of variance. Fraction 2 compared with other fractions ($P<0.01$); the difference between other fractions no significant ($P>0.05$).

**Table 5. Comparison of antigen specific activity between HPLC fraction 2 and TF-HSV-1**

| TF preparations | Antigen | LAII %[a)] |
|---|---|---|
| Fraction 2 ($10^{-8}$ U/ml) | HSV-1 | 17.9 ± 1.4 |
| TF-HSV-1 ($10^{-3}$ U/ml | HSV-1 | 16.7 ± 1.6 |

a) Mean ± SE for 6 and 4 independent experiments, respectively by group t-test analysis ($P>0.05$).

movement of a surface electrode on the gel from the anode to cathode. The gel was stained by silver staining (Fig. 3). Only a single band of the fraction 2 was found on the IEF gel, which PI was pH 4.48.

The results demonstrate that a highly purified TF active component specific for HSV-1 (PTFC) was obtained by RP-HPLC purification. The RP-HPLC employed here was not only a powerful mean for removing impurities from TF, but also allowed the direct biochemical identification of PTFC.

In conclusion, the isolated and purified HSV-1 specific TF active component has a hydrophilic property and a PI of pH 4.48 by affinity chromatography and RP-HPLC. The two-step purification procedure applied here has shown significant advantages: a) specificity and high resolution suitable for isolation and purification of a very small amount of TF active component from the starting dialysate with complex materials; b) avoiding unnecessary purification steps which will involve the loss of valuable material. The above results will allow us to obtain adequate amounts of PTFC for further analyzing and understanding the characterization of HSV-1 specific TF.

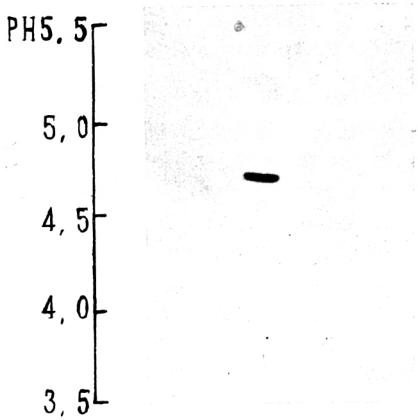


**Fig. 3**
Isoelectric focusing of HPLC fraction 2
pH 3.5 - 5.5, 5 °C, 5 W, 3 hr.

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