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NATURE AND ANTIGEN-SPECIFIC ACTIVITIES OF TRANSFER FACTOR AGAINST HERPES SIMPLEX VIRUS TYPE 1

L. L. HUANG, C. Z. SU, Z. F. WAN

Dpt. of biochemistry, Fourth Military Medical College, Xian, China

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Summary. — Transfer factor specific for herpes simplex virus (HSV) type 1 (TF_{HSV-1}) was prepared from splenic cells of HSV-1 immunized mice. Protection was transferred with TF_{HSV-1} to nonimmune mouse recipients. The TF_{HSV-1} injected mice had a higher survival rate after lethal HSV-1 challenge as compared to mice injected with a nonspecific transfer factor ($P < 0.05$). ^{51}Cr-labelled leukocyte adherence inhibition (^{51}Cr-LAI) test was used to demonstrate the specific activity of transfer factor in vitro. Only leukocytes incubated with TF_{HSV-1} exhibited significant adherence inhibition ($P < 0.01$) to HSV-1 antigen, but not to control antigen. Specific activity component of TF_{HSV-1} (STFc) was separated by affinity adsorption with the antigen. Activity of STFc in ^{51}Cr-LAI test was significantly higher than that of TF_{HSV-1} ($P < 0.01$). Ratio activity of STFc in protective host immunity was 16 times as much as that of TF_{HSV-1}. STFc was analysed by high performance liquid chromatography, thin layer chromatography and isoelectric focusing in the polyacrylamide gel. Results revealed that STFc appeared to be a polypeptide with a molecular weight of about 12,870 dalton.

Key words: transfer factor, herpes simplex virus type 1, antigen-specific component, leukocyte adherence inhibition, protective host immunity

Introduction

Herpes simplex virus type 1 (HSV-1) infection is a major cause of acute necrotizing encephalitis (White and Taxy, 1983). Although new antiviral agents such as acyclovir hold some promise for the management of herpes infections, no satisfactory treatment is currently available. The immune response is capable of interacting with a HSV-1 infection, since the course of infection in immunosuppressed patients is usually more severe (Rajčáni *et al.*, 1974; Lopez, 1983). The finding that athymic (nu/nu) mice are susceptible to intradermal herpes infections implies that T cell mediated responses are essential for antiviral immunity. Adoptive transfer of immune spleen cells could protect the recipient against a lethal herpes infection, the protective T cells being $Lyt1^{+}2^{-}$ (Nash and Wildy, 1983).

Transfer factor (TF) is a dialyzable component(s) of leukocyte lysate that is capable of transferring delayed type hypersensitivity. Steele *et al.* (1976) demonstrated the efficacy of human TF in preventing death from HSV-1 induced fatal infection in an animal model. Antigen specific TF has been shown to have excellent prophylactic value in protection from varicella in children with leukaemia who are immunosuppressed by chemotherapy (Steele *et al.*, 1980). Viza *et al.* (1983) have shown the beneficial effect of bovine TF on herpes patients. Dwyer (1983) using specific human TF confirmed this observation in a controlled trial. Viza *et al.* (1986) have also reported that HSV-specific TF of bovine origin protects mice against the corresponding HSV virus, whereas the injection of a nonspecific TF fails to protect against HSV-1 challenge.

The chemical nature of the active substance of TF has not yet been defined. The mechanism of action is virtually unknown. A major controversy surrounding TF is the issue of specificity. This report is to demonstrate the antigen-specific activities and the potential for protective host immunity of TF against HSV-1. It was tempting to isolate and identify the effective fraction(s) contributing to these antigen-specific activities.

Materials and Methods

Preparation of TF. We used male LACA mice aged 8 to 12 weeks. All animals were raised in our animals care facility. Mice were sensitized to HSV-1 by intraperitoneal (i.p.) inoculation with living HSV-1 strain SM-44 in 199 medium, which was generated in a baby hamster kidney (BHK) cell line. Control mice received the 199 medium alone by the same schedule. HSV-1 immunized mice exhibited marked footpad swelling when receiving to HSV-1 antigen. Pathological examination of the swollen footpad skin revealed a profuse mononuclear infiltration in the area of induration. TF was prepared from splenic cells coming from either control mice (TFn) or HSV-1 immunized animals ($TF_{HSV\text{-}1}$) removed 4 weeks after immunization. Briefly, splenic cells were disrupted and the lysate was subjected to a normal pressure dialysis by using tubing with a 12,000—14,000 molecular weight (M.W.) exclusion size (Spectrum Medical Industries Inc., U.S.A.) into sterile distilled water at 4 °C for 48 hr. The dialyzable fraction was concentrated by lyophilization and reconstituted in 0.9% NaCl solution (NS) to a concentration revealing absorbance 8.0 at 260 nm equivalents (1 unit of TF).

^{51}Cr-labelled leukocyte adherence inhibition assay. A modification of the method of Tsang *et al.* (1980) was used. Mononuclear cells were obtained from spleen of normal male LACA mice aged 8 to 12 weeks, weighing 18 to 20 g. Cell pellet was diluted in GNK solution (glucose 1 g/l, NaCl 4 g/l, KCl 4 g/l in Tris 1.8 g/l, pH adjusted to 7.4 with HCl) to a concentration of $2.5-4\times$ $\times 10^7$ cells/ml. Then 5 μCi of ^{51}Cr was added to 8×10^6 cells suspension and incubated at 37 °C for 1 hr. Cell pellet was washed 3 times in GNK solution. To each glass tube (flat bottom, ∅ 11 mm) was added 5×10^5 of ^{51}Cr-labelled cells in 0.1 ml medium 1640 (RPMI) enriched with 5% foetal bovine serum. Then 50 μl of antigen and 50 μl of TF were added to each test tube. The antigens used were purified by ultracentrifugation. Medium 1640 instead of antigen and TF was added to control and blank tubes. Cell suspension was incubated at 37 °C for 2 hr, then the adherent cells were harvested and counted. The results were expressed as leukocyte adherence inhibition index (LAII). All data were analysed for statistical significance by the Student's test.

$$\text{LAII} = \frac{\text{MAR of control group} - \text{MAR of test group}}{\text{MAR of control group}} \times 100\%$$

MAR = mean adherence rate

Table 1. Antigen-dependent activity of TF_{HSV-1} assayed in ^{51}Cr-LAI test[a]

Experiment	TF_{HSV-1} + HSV-1 Ag	HSV-1 Ag only	TF_{HSV-1} only	TF_{HSV-1} + Control Ag	TFn + HSV-1 Ag	TF_{HSV-1} + Measles virus Ag	TF_{HSV-1} + Japanese encephalitis virus Ag
1	18.85	3.44	−1.27	0.56	4.46	−0.64	3.98
2	17.12	1.32	3.41	−1.55	−0.23	4.56	−5.98
3	20.40	2.34	2.70	−6.86	0.87	−0.84	3.73
4	18.97	1.44	3.08	−1.19	−5.18	−1.00	4.98
5	23.44	4.96	2.96	5.25	−2.81	5.64	5.59
6	24.91	2.74	1.97	6.51	−1.01	1.85	6.15
Mean	20.62	2.71	2.14	0.45	−0.65	1.60	3.08
SE	1.22	0.56	0.71	2.00	1.34	1.20	1.85
P value[b]		<0.01	<0.01	<0.01	<0.01	<0.01	<0.01

[a]) The results were expressed as LAII. Final concentration of TF was 10^{-5} unit/ml and amount of antigen (Ag) was 10^{-4} μg protein/tube in the experiment.

[b]) Compared with TF_{HSV-1} + HSV-1 group.

Table 2. Antigen-dependent activity of STFc assayed in ^{51}Cr-LAI test[a]

Exp.	LAII[b]	
	STFc + Control Ag	STFc[c] + HSV-1
1	0.08	22.70
2	−1.89	25.38
3	4.14	26.28
4	4.17	30.81
5	−0.99	26.30
Mean ± SE	1.10 ± 1.28	26.29 ± 1.31
P value[d]	<0.001	

a) Final concentration of STFc was 0.25×10^{-6} unit/ml and amount of antigen was 10^{-4} μg protein/tube in the experiments
b) LAII = leukocyte adherence inhibition index
c) STFc = specific transfer factor component
d) By the Student's test

Transfer of protection. BALB/c mice of either sex, 19-day-old, weighing 7.5−8.5 g were used. TF was administrated intraperitoneally. Then 72 hr later the mice injected with TF were challenged by i.p. route with 5 LD_{50} of HSV-1 strain SM-44. Animals received TF twice weekly up to death of the survivors for 28 days. The survival time and percentage of animals surviving day 28 after challenge were determined and compared with that of a sham challenged control mice. The Logrant test in survival analysis (Feng,1982) was used to determine the significance of the results.

Isolation of specific transfer factor component by affinity adsorption with antigen. HSV-1 antigen was purified by ultracentrifugation. Immunoadsorbent was prepared as described (Avrameas and Ternynck, 1969). Carrier in the adsorbent was bovine serum albumin (BSA). Final concentration of BSA was 50 mg/ml. TF_{HSV-1} was incubated with the adsorbent overnight by stiring at 4 °C. Immunoadsorbent was then washed with PBS (KH_2PO_4 6.125 g, NaOH 1.025 g, NaCl 8.775 g/l, pH 7.0) at 2−4 °C until the effluent absorbence at 260 nm and 280 nm became negligible. The adsorbent was then eluted with PB (KH_2PO_4 6.125 g, NaOH 1.025 g/l, pH 7.0) at 20 °C for 30 min. The eluant collected was designated specific transfer factor component (STFc). TFn did not bind to adsorbents containing HSV-1 antigen. TF_{HSV-1} did not bind to adsorbents consisting of BSA alone.

Results and Discussion

Immune activity of specific transfer factor and its component (STFc) in ^{51}Cr-LAI assay

The ^{51}Cr-labelled leukocyte adherence inhibition test index was used to demonstrate the specific activity of TF_{HSV-1} in vitro (Table 1). Leukocytes obtained from nonimmunized mice were divided into eight groups as follows: control without TF and antigen; TF_{HSV-1} and HSV-1; TF_{HSV-1} alone; HSV-1 alone; TF_{HSV-1} and control antigen obtained from normal BHK cells; TFn and HSV-1; TF_{HSV-1} and measles virus; TF_{HSV-1} and Japanese encephalitis virus. When normal leukocytes were incubated with TF_{HSV-1} and HSV-1 antigen, the leukocyte adherence inhibition index was signifi-

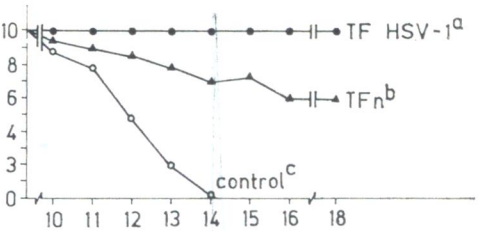

Fig. 1.
Host protection to a challenge infection TF HSV-1 and TFn[d]
[a]) VS c = $P < 0.01$
[b]) VS c = $P < 0.01$ by Logrant test
[c]) VS b = $P < 0.05$
[d]) TF was administrated in dose equivalent 300 µg polypeptide per mouse. Control mice were injected with NS instead of TF. Challenge was 5 LD50 of HSV-1 SM-44 strain; 10 mice/group.
Abscissa: survival time (days); ordinate: number of survivors.

cantly higher than that of control group ($P < 0.01$). In the former, LAI index was 20.62 ± 1.22 (mean $\pm$SE). Leukocytes exposed to nonspecific TFn did not respond to HSV-1 in vitro, but those exposed to TF_{HSV-1} showed no adherence inhibition when tested with control antigen, measles virus, or Japanese encephalitis virus antigen. Leukocyte adherence inhibition (LAI) was not seen in the group of TF_{HSV-1} or HSV-1 alone. The difference between LAII to HSV-1 of TF_{HSV-1} recipients as compared with other experimental groups tested was highly significant ($P < 0.01$). The results demonstrated that TF_{HSV-1} could transfer specific cell-mediated immunity to non-immune leukocytes. LAI was also seen when normal leukocytes were incubated with specific transfer factor component (STFc) and HSV-1 antigen, but not with control antigen (Table 2). Antigen-dependent LAI activity of STFc was compared with that of TF_{HSV-1}. In the experiments final concentration of STFc and TF_{HSV-1} was 0.25×10^{-6} unit/ml and 10^{-5} unit/ml, respectively. The results revealed that LAII ($27.08 \pm \pm 1.33$, mean $\pm$SE, $n = 6$) of STFc group was significantly higher than that (20.62 ± 1.22, mean $\pm$SE, $n = 6$) of TF_{HSV-1} group ($P < 0.01$). It has been suggested that specific activity of STFc assayed in ^{51}Cr-LAI test was 40 times as much as TF_{HSV-1} ($10^{-5}/0.25 \times 10^{-6} = 40$).

Protective host immunity to HSV-1 was transferred to non-immunized BALB/c mice with TF_{HSV-1} (Fig. 1). Host survival to a lethal HSV-1

Table 3. Resistance to HSV-1 in BALB/c mice receiving either TF_{HSV-1} or STFc

Mice injected with		Estimate reference	SD
TF_{HSV-1}	0 µg	8.590178	7.854479
TF_{HSV-1}	60 µg	2.699405	5.560581
TF_{HSV-1}	120 µg	−0.400369	5.473125
TF_{HSV-1}	180 µg	−1.221997	5.471988
TF_{HSV-1}	240 µg	−1.831095	5.490471
TF_{HSV-1}	300 µg	−6.633318	0.000000
STFc	15 µg	−2.797838	5.563669

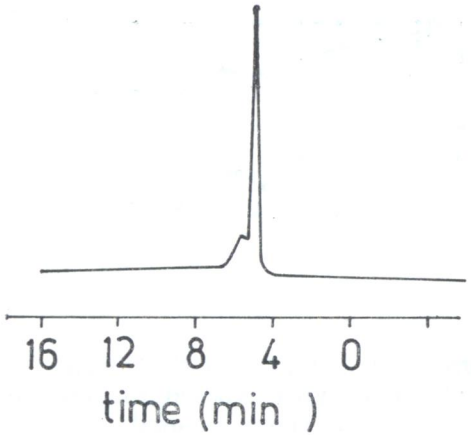

Fig. 2.
HPLC of STFc on a 0.45 × 24 cm column of octadecyl silane
Chromatography was performed at a flow rate of 0.62 ml/min under 70 BAR. Methanol/0.1% phosphate acid in water (1/1) was used as eluent. Absorbance at 280 nm was determined.

challenge was seen in TF_{HSV-1} injected mice. The percentage of survivors was 100%. All ten control animals not receiving TF died. TFn recipients had a lower percentage of survivors (60%) than the TF_{HSV-1} recipients ($P < 0.05$).

STFc obtained by affinity adsorption could also prevent fatal disseminated HSV-1 infection in mice. STFc was injected in dose equivalent 15 μg polypeptide per mouse. Administration schedule of STFc is the same as TF_{HSV-1}. Four in five mice receiving STFc survived to 28 days. The results demonstrated that STFc had the potential to protect the host infected with HSV_{-1}. Host protection provided by STFc and TF_{HSV-1} were compared. All data were analysed by Cox model in survival analysis (Table 3). The results revealed that the activity of STFc assayed for host protection test was 16 times as high as TF_{HSV-1} (240/15 = 16).

Chemical characterization of the specific transfer factor component

The specific transfer factor component was analysed by high performance liquid chromatography (HPLC). Anion exchange chromatography of STFc through Aminex A-27 (Bio-Rad) utilizing a Pye unicam's model LC3 high pressure liquid chromatograph yielded one UV absorbing peak. Chromatography was performed on a 0.45 × 25 cm column at a flow rate of 1 ml/min under 90 BAR. As eluent 5 mmol/l amonium acetic acid was used. STFc was further purified by high pressure reverse-phase liquid chromatography (Fig. 2). An obvious UV (220 nm and 280 nm) absorbing peak was obtained

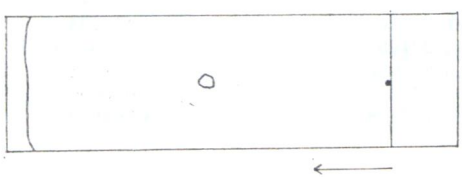

Fig. 3.
Thin layer chromatography of STFc on 6 × 20 cm silical gel HF_{254} plate
There was a clear Fluram-reactive spot (Rf = 0.51).

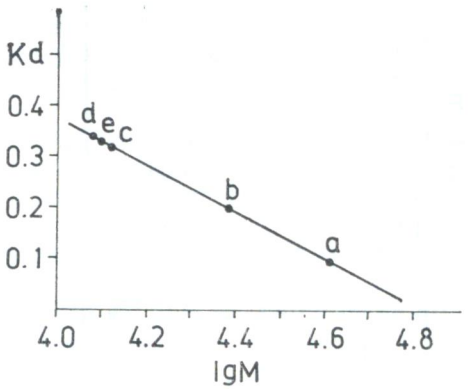

Fig. 4.
Determination of molecular weight of STFc on Sephadex G-50 (medium)
a) Ovalbumin; b) Chymotrypsinogen A; c) Ribonuclease A; d) Cytochrome C; e) Specific transfer factor component.
Kd = Ve − Vo/Vi
lg M = log M. V.

using a solvent system of H_3PO_4 and methanol on a column bed packed with ODS resin (YWG-$C_{18}H_{37}$, China).

Thin layer chromatography (TLC) of specific transfer factor component was performed on 6×20 cm silical gel HF_{254} plate (Merck, Fed. Germany). Developing solvent system was n-butanol/glacial acetic acid/water (26/6/10). About 30—40 μl of the sample containing 30 to 40 μg of polypeptide were spotted per plate. At the end of this development the plate was dried, scanned with UV 254 nm and then sprayed with 0.05% fluorescamine in acetone. No UV(254)-absorbent spot was seen, and there was clearly visible a Fluram-reactive spot (Fig. 3).

Isoelectric focusing (IEF) in thin layer polyacrylamide gel of STFc was performed employing pH gradients of 3.5—10. At the completion of IEF, the gel was then stained by Coomassie Blue R250 (Fluka) and destained. The result showed only one band containing STFc.

Determination of molecular weight of the specific transfer factor component was performed on 2.2×50.3 cm column of Sephadex G-50 (Pharmacia) at a flowrate of 0.38 ml/min. The M.W. callibration kit was provided by Pharmacia Co.; 0.9% NaCl solution was used as eluent. The results revealed a M.W. of 12,870 dalton (Fig. 4) for STFc.

Chemical nature of specific transfer factor component was analysed by reactivity with Coomassie Blue G250 (Branford, 1976), fluorescamine, orcinol, diphenylamine and sulphosalicylic acid (Table 4). Amino acid con-

Table 4. Chemical characteristics of specific transfer factor component

Coomassie Blue G250	Positive
Fluorescamine	Positive
Orcinol	Negative
DNA Content	No
Free Amino Acid	No
20% Sulphosalicylic Acid	Negative

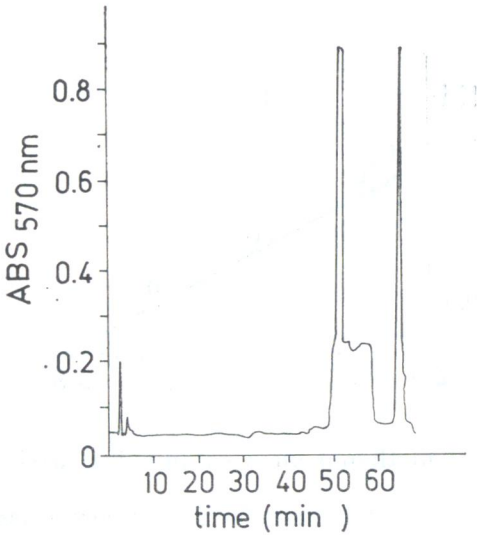

Fig. 5.
Determination of free amino acid of STFc
The determination was performed by Beckman Amino Acid Analyzer. The result showed STFc did not contain free amino acid.

tents of STFc were assessed with a Backman Amino Acid Analyzer (Figs. 5, 6). STFc did not contain DNA and free amino acid; the reactivity of STFc with orcinol was negative. Lysate of STFc contained the following amino

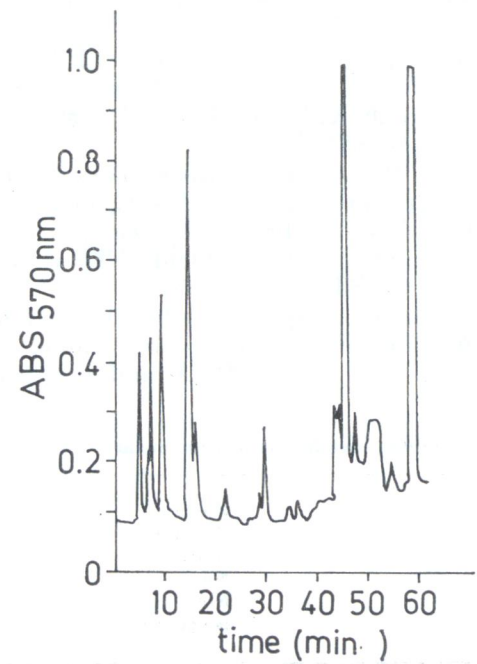

Fig. 6.
Amount of amino acid in STFc lysate
The amount was assessed with a Beckman Acid Analyzer. STFc lysate contained 16 kinds of amino acids.

acids: aspartic acid (asparagine), threonine, serine, glutamic acid (glutamine), proline, glycine, alanine, valine, methionine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine.

The following conclusions were drawn from these experiments:

1. TF_{HSV-1} could transfer specific cell-mediated immunity to nonimmune leukocytes in ^{51}Cr-LAI test.

2. Protective host immunity was transferred to non-immunized mice with TF. Survival rate (100%) of mice receiving TF_{HSV-1} was higher than that (60%) of mice receiving TFn ($P < 0.05$). Effect of TF_{HSV-1} was dose-dependent.

3. Specific transfer factor component was separated from TF_{HSV-1} by affinity absorption to HSV-1 antigen.

4. STFc also had antigen-dependent activity and the potential to protect the host. The activity of STFc in immune reaction was higher than that of TF_{HSV-1}.

5. STFc appeared to be a polypeptide with M.W. of about 12,870 dalton.

6. ^{51}Cr-LAI test is suitable for detecting TF specific activity.

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