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DIALYSABLE SPECIFIC TRANSFER FACTOR IN MICE IMMUNIZED WITH ATTENUATED LANGAT VIRUS FROM THE TICK-BORNE ENCEPHALITIS COMPLEX: GENERATION, ACTION AND QUANTITATIVE ASSAY

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Summary. — Cytolytic T lymphocyte assay was developed in order to measure the response of inbred C3H mice to dialysable specific transfer factor (STF), induced in subadult outbred mice by one shot immunization with the attenuated Langat virus. The first STF activity in mice splenic leukocytes was detected between 48-72 hr after virus administration. The conversion of splenic T-cell cytotoxic response in C3H mice in vivo occurred between 15-21 hr after STF administration. The killing activity of T-cells, induced by STF, showed cross-reactive traits within the genus *Flavivirus*. STF, given prior to the live virus, augmented the specific cytolytic T-cell response. In the live virus-primed mice the booster effect was markedly enhanced when administration of STF preceded the second immunization dose. In the serum of STF recipients, interferon was irregularly detected attaining low levels for short time periods. Temperature of 56 °C for 60 min abolished the activity of least 10^4 murine STF units, temperature of 37° C lowered after 24 hr this activity by 3 $\log_{10}$units. Chromatography of the dialyzed leukocyte lysate on Sephadex G-25 column yielded usually five peaks. The second peak showed an increased content of ribose-bound and protein materials and, as a rule, a relatively concentrated STF activity.

Key words: *transfer factor*; *specific activity*; *cytotoxic T lymphocytes*; *Flaviviruses*

Introduction

Lawrence's original observations (1955, 1974) on the transfer of the cutaneous delayed type hypersensitivity response by low-molecular components (m.w. 10.000) of human leukocyte (Le) lysates (transfer factor, TF) were considerably extended during the recent years (see e. g. Khan *et al.* 1979). Dialysates of lyzed leukocytes (DLL) were shown to contain several immune response regulators, falling apparently into two categories, i. e. antigen-dependent and antigen-independent activities. Whether these effects are produced by identical or different components of the dialysate, has not been definitely elucidated (Petersen and Kirkpatrick, 1979). The lack of a meaningful in vitro assay or of a reliable experimental animal model, impaired for a long time the investigations on biochemical identity, unique biological properties and mechanisms of TF action, such as conversion of the cell-mediated immune (CMI) response in non-immune recipient according to the donor's immunological experience. The recognized theoretical and clinical imporance of TF warrants a deeper exploration of its efficacy to influence infections caused by viruses, belonging to various taxonomic groups (e. g. Valdimarsson *et al.*, 1979, Hamblin 1979).

In efforts to meet such a demand, we attempted to develop a relatively versatile model, allowing a quantitative approach to the study of STF. In pilot experiments, outbred mice, one shot-immunized by a live attenuated virus from the tick-borne encephalitis (TBE) complex, were shown efficient STF producers. The xenogeneic STF has been demonstrated in ^{51}Cr-release assay by generating cytotoxic T lymphocyte (Tc) response (Mayer *et al.*, 1980). The present report deals with the kinetic of STF formation and quantitation of STF action.

Materials and Methods

Viruses. Langat virus (TBE complex) variant "14" derived from the E5 strain (Mayer 1975) was used. One ml of the 10% brain suspension contained 8.1×10^7 PFU as detected in pig kidney (PS) cells, which corresponded to 7.3 log i.c. LD_{50}/ml in outbred specific pathogen free suckling mice (s.m.). The E5 "14" variant was not virulent when administered subcutaneously (s.c.) to subadult mice. Strain "204" of the TBE (western subtype) virus, was obtained from the WHO Collaborating Centre for Arboviruses, Institute of Virology, Bratislava. The stock virus suspension titered 9.1×10^7 PFU/ml in PS cells or 8.7 log i.c. LD_{50} and 7.5 log s.c. LD_{50} per ml in s.m. The 17D strain of the Yellow fever virus, originated from a commercial vaccine (Burroughs Wellcome and Co., London, Leukosis free lot 4512). The stock virus suspension titrered 6.8×10^6 PFU per ml in PS monolayers. West Nile virus, strain "99" isolated from mosquitos in Western Slovakia (Labuda et al., 1974) had undergone 12 s.m. brain passages. The stock virus suspension titered 7.5 log i.c. LD_{50} per ml, corresponding to 1.1×10^8 PFU ml per in PS monolayers. Dengue type 1 virus, the prototype Hawaian strain, was obtained as the "204" virus. The stock virus suspension titered 7.5 log i.c. LD_{50}, or 7.2×10^7 PFU per ml. Sindbis virus, obtained as mentioned above, titered 7.7×10^7 PFU per/ml in chick embryo cell cultures.

Mice. Specific pathogen-free outbred mice, strain "ICR" weighing 10—12 g, were used. Cohort mice were given one immunizing dose comprising 10^4 PFU of the Langat virus, either by s.c. (0.1 ml) or intraperitoneal (i.p., 0.25 ml) or intravenous (i.v., 0.1 ml) routes. Matched controls, received either 0.14 mol/l NaCl, or diluted uninfected s.m. brain suspension, by each route. The same animals were used for titration of flaviviruses. As STF recipients, male C3H/Cbi/BOM/H-2^k/ mice were used purchased from the Šumice breeding farm when 4—5 weeks old.

^{51}*Cr-release assay.* To test the dialyzed leukocyte lysate (DLL) or other materials containing STF activity, splenic Le from C3H mice were used as effector cells. L929 cells, syngeneic with C3H mice, were used as target cells. They were grown in basal Eagle's medium (BEM), supplemented with 5 per cent foetal calf serum and seeded in tubes (3×10^5 cells). The 48 hr old, confluent STF activity splenic Le from C3H mice were used as effector cells. L929 cells, syngeneic with C3H mice, were used as target cells. They were grown in basal Eagle's medium (BEM), suplepplemented with 5 per cent foetal calf serum and seeded in tubes (3×10^5 cells). The 48 hr old, confluent monolayers were infected at an input multiplicity of 2 PFU per cell with TBE virus or other viruses, as indicated. ^{51}Cr-release was performed as previously described (Gajdošová *et al.*, 1981). Different experimental groups were compared for statistical significance of results obtained by two-tailed Student's test.

Preparation of dialyzed murine splenic leukocyte lysates. By 19—29 days after immunization, donor mice splenic Le were harvested from the minced spleens; crude cell suspension was filtered through sterile wool column in 15 × 1.5 cm glass cylinders. The cells were washed in PBS treated with 0.87% HN_4Cl for 10 min, spun, resuspended in bidistilled water, counted and frozen at —18° C. Individual cell batches came from 100—300 mice. Each day splenocytes were prepared from 30—35 animals. When the whole cohort was proceeded, the splenic cell suspensions were pooled and after adding pancreatic DNase (SERVA) and $MgSO_4$ (Rifkind *et al.*, 1977), the whole pool was frozen and thawed ten times in dry ice-alcohol bath. The resulting cell lysate was dialysed against 20 volumes of bidistilled water for 48 hr at 4° C under continuous stirring. If not indicated otherwise, after the first 24 hr the resulting dialysate (A) was removed and replaced by an equal volume of chilled water. After further 24 hr, the latter dialysate (B) was pooled with the former, shell-frozen and concentrated by lyophilization. The dry substance was stored at —30° C in tightly stoppered vials. Before use, the substrate was redissolved in bidistilled water. As will be outlined later, 20 mg of the freeze-dried substance was considered arbitrarily as one unit of DLL. This amount corresponded roughly to the amount of dialysable material yielded by 3×10^8 splenic cells, obtained from three spleens. The DLL from placebo-administered mice was prepared in a similar way. The DLL unit from various batches varied in its STF activity.

Titration of the antigen-specific TF activity. The capacity of STF in the DLL to "instruct" the killer T cells to destroy the TBE virus antigen-bearing cells was titrated as follows. Individual dilutions of the tested material were immediately inoculated i.v. or i.p. to three recipient C3H mice; thereafter, the spenocytes were isolated, pooled and used as effector cells in ^{51}Cr-release cytotoxic assay. The highest dilution, causing generation of specific Tc cells in the recipient spleens releasing ^{51}Cr from target cells in a degree, differing significantly ($P < 0.001$) from the spontaneous ^{51}Cr-release was considered to represent the STF end-point activity (1 unit). Under standardized conditions, the ^{51}Cr-release values considered positive were not lower than 30%, whereas the spontaneous ^{51}Cr-release from infected target cells ranged between 20—24%. The titre of the STF activity was expressed as $\log_{10}$ value of the end-point dilution and calculated for one ml of the undiluted starting material. As mentioned before, the amount of standard starting material containing one DLL unit corresponded to 20 mg of the lyophilized dialysable substance, reconstituted in 1 ml diluent.

Interferon assay. Mice were bled by puncture of orbital sinus. At intervals, the blood was pooled from three animals and serum preserved frozen until use. The diluted serum were added to L929 cell monolayers, challenged after overnight incubation with 100 CPD_{50} of the encephalomyocarditis virus (Lackovič and Borecký, 1965). A sample of standard interferon (IFN) was kindly provided by dr. V. Lackovič, Institute of Virology, Bratislava.

Gel filtration. In reported experiments, 3.25—5.20 units of DLL, dissolved in one ml of sterile bidistilled water were applied to a 2.5 × 90 cm column of Sephadex G-25 fine (Pharmacia, Uppsala). Bidistilled water was used as the eluent and the flow rate was adjusted to 25 ml/hr. Elution was monitored by recording the absorbence at 260 and 280 nm. Individual fractions of the eluate from Sephadex G-25 column were pooled and freeze-dried.

Determination of protein and RNA content. The amounts of protein in DLL or in its column fractions were determined by the procedure of Lowry *et al.* (1951). Bovine serum albumin was run as a standard. RNA or ribonucleotide contents were determined as purine-bound ribose by the technique described by Mejbaum (1939) using orcinol and $FeCl_3$ (orcinol-reactive material, ORM).

Enhancement of E-rosette formations by murine TF. Ly were isolated from 4 ml aliquots on Ficol-Metrizoate gradient by centrifugation at 400 × g for 40 min. Washed Ly, at a 4×10^6 per ml concentration, were mixed with 0.25% trypsin and incubated for 30 min at 37 °C (Holzmann and Lawrence, 1977). Then, to 0.9 ml of Ly suspension was added 0.05 ml of TF-containing or control material supplemented with 0.05 ml of foetal calf serum. In control tubes, sterile distilled water was added to Ly instead of the TF material. All tubes were incubated for 3 hr at 37 °C, washed three times and resuspended to the original volume; 0.2 ml of the Ly suspension was mixed aa with 1% sheep red blood cells (SRBC), incubated for 10 min at 37 °C and spun down at 200 × g for 5 min. The tubes were then kept overnight at 4 °C. One droplet of 0.004% acridine orange was added and the pellet gently resuspended. Cells bearing at least 3 SRBC were regarded as rosettes, always 200 Ly were scored. Human. DLL (obtained from Dr. J. Pekárek, Institute of sera and vaccines, Prague) was used as positive control.

Table 1. Cytolytic activity of STF primed effector spleen cells induced by DLL in C3H mice demonstrated on syngeneic virus infected target cells

DLL*	Per cent of ^{51}Cr-release (means $\pm$ SD)**	P
undiluted	53.22 ± 2.9	< 0.001
1 : 10	49.51 ± 0.7	< 0.001
1 : 10.000	32.11 ± 1.1	< 0.001
1 : 100.000	26.23 ± 1.0	> 0.02
DLL from splenocytes of non-immunized mice	18.96 ± 0.56	> 0.02
None	22.45 ± 0.4***	> 0.02

* DLL containing STF activity was concentrated by lyophilization; 20 mg of the freeze dried substance reconstituted with 1 ml of distilled water; 0.1 ml given by i. v. route to C3H mice. DLL had been prepared from spleens of mice 21 days after immunization with the live attenuated Langat virus.

** Targets cells were in contact with effector cells for 18 hr;

*** Spontaneous ^{51}Cr-release

Results

STF in mice immunized with the attenuated Langat virus

Splenic Le from C3H mice, which had received i. v. 0.1—0.00001 units of DLL (prepared by immunization of specific pathogen-free mice with a single dose of Langat virus), exerted significant cytotoxic activity when brought into contact with the ^{51}Cr-labelled, TBE virus-infected L929 cells, syngeneic for the H-2^{k} allele. This was not observed when splenocytes were used from C3H mice, which had received DLL prepared from spleens of placebo-inoculated mice (Tab. 1).

Seven different batches of DLL tested contained $4 \times 10^3 - 10^5$ units of the STF activity per DLL unit. Chromatographic fractions (see later), showing the highest ratio of absorbance at 260 and 280 nm, contained $4 \times 10^4 - 10^5$ STF units per 20 mg of lyophilized eluate. The Tc response of C3H mice was by about 10 per cent higher after three DLL doses, administered on subsequent days, than after two doses. No significant difference in Tc response was observed between mice receiving one or two DLL doses. Batches

Table 2. Restoration of SRBC rosettes with trypsin-treated Ly by DLL

Lymphocytes				DLL		
Native	Treated	Treated plus MEM	Treated plus DLL	Concentration	DLL/Ly ratio	Origin
56.6*	16	11	25	4.10^{6}**	10/1***	human
66	7.5	8.5	14.5	6.10^{6}	24/1	murine
78.5	34.5	45.3	60	3.10^{7}	60/1	murine
54	28	7.5	70	3.10^{7}	60/1	murine

* = per cent of rosettes

** = number of cells which the given amount of DLL was prepared from

*** = number of cells being the source of DLL calculated per one Ly

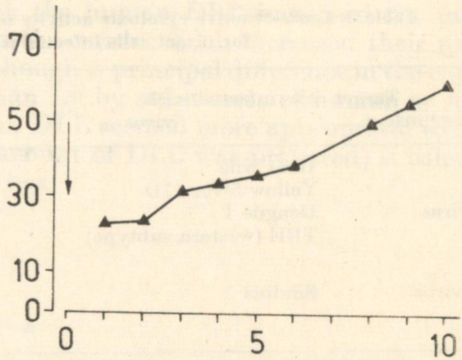

Fig. 1.
Induction of SPF in splenocytes of inbred C3H mice inoculated with attenuated Langat virus
Abscissa: days post inoculation of 10^4 PFU of the virus; ordinate: per cent of ^{51}Cr-release

of DLL obtained after 48 hr dialysis against 20 volumes of bidistilled water at 4 °C and by one exchange after 24 hr (dialysates A and B, see Materials and Methods) were compared. About 99 per cent or more STF activity was present in the A material. This is illustrated by analysis of the representative DLL batch No. 10. One ml of the dialysate A (from 300 ml volume) contained 0.66 mg of the dry substance; alternatively, one mg of the freeze dried A material contained 104 μg of protein and 8 μg of ORM, but in the same amount of the B material 0.04 mg dry substance, 275 μg/mg of protein and 20 μg of the ORM were found. STF activity was studied separately in 10 mg of each, A and B substrate. 10^4 STF units were found in the investigated amount of the A lyophilizate and 10^2 in the B lyophilizate. Similarly, 90—99.9% of the STF activity was observed to be present in the A material from DLL batches No. 12 and 13, 100% in the batch No. 14.

Although a relatively standard method of DLL preparation was used throughout, the ORM and protein contents ranged among individual preparations (174—240 μg of ORM and 1140—1340 μg of peptide per 20 mg of lyophilized DLL). Nevertheless, the STF activity in various DLL batches was found to be not lower than 4×10^3 STF units per one DLL unit. The varying ORM and protein quantities observed in individual DLL batches are a good reason for separate estimation of STF activity expressing it not

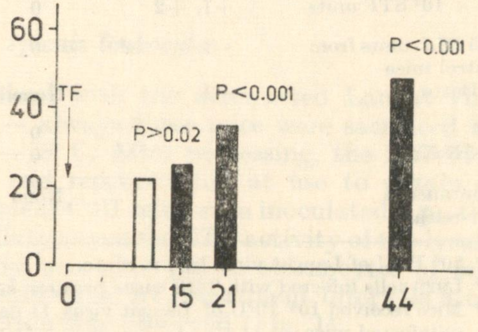

Fig. 2.
Expression of STF activity by the cytotoxic response of splenocytes from C3H mice administered 0.1 ml of DLL (0.1 DLL unit comprising 10^4 TF units)
Abscissa: hr after TF administration; ordinate: per cent of ^{51}Cr-release
□ spontaneous ^{51}Cr-release from the target cells
■ specific ^{51}Cr-release from the target cells

Table 3. Cross-reactive cytotoxic activity of SPF primed effector spleen cells for target cells infected with flaviviruses

Target cells infected with genus	virus	Per cent of ^{51}Cr-release (means ± S. D.)
Flavivirus	West Nile	54.65 ± 1.8*
	Yellow fever 17D	56.25 ± 2.1
	Dengue 1	57.95 ± 1.5
	TBE (western subtype)	57.40 ± 2.1
		22.87 ± 0.5**
Alphavirus	Sindbis	26.65 ± 1.1
None		22.99 ± 0.9***

* DLL prepared in mice immunized with Langat virus; ** DLL from placebo-given mice; *** spontaneous ^{51}Cr-release;
Notice: The difference between cytotoxic activity of the STF primed splenic lymphocytes in target cells infected with various flaviviruses is not significant in contrast to the alphavirus-infected targets.

only in weight amounts of DLL or in splenocyte number equivalents as frequently used in literature.

Effect of TF on restoration of trypsin damaged T lymphocyte receptors for SRBC

STF activity was studied for its capacity to accelerate the restoration of trypsin-damaged T Ly membrane receptors for SRBC. The trypsin treatment lowered the number of rosettes forming Ly in average by 67 per cent

Table 4. Enhancing effect od DLL containing SPF on generation of specific T cells in naive and immunized C3H mice

STF i.p.	Administration day	Virus infection on day*	% ^{51}Cr-release (means ± S.D.) naive mice Exp. A.	naive mice Exp. B.	immunized mice***
2.5 × 10^4 STF units	−1	0	80.37 ± 4.06**	38.25 ± 1.45	70.02 ± 2.30
2.5 × 10^4 STF units	+1, +2	0	56.50 ± 1.94	ND	ND
0.25 DLL units from control mice	−1	0	ND	22.62 ± 0.45	35.33 ± 1.32
Detto	−1	None	20.90 ± 2.01	ND	ND
Nil		0	48.12 ± 2.35	22.81 ± 1.63	51.09 ± 5.33
0.14 N NaCl		0	ND	25.86 ± 1.88	38.62 ± 0.35
Spontaneous ^{51}Cr-release		None	23.18 ± 1.21 ND	20.41 ± 0.73 23.97 ± 1.21	

* 10^5 PFU of Langat virus by i.v. route
** L929 cells infected with TBE virus (western subtype)
*** Mice received 10^4 PFU of Langat virus 11 days before. Placebo = 5 mg dried DLL from uninfected mice

(range 49—89%). Incubation with the human DLL increased the number of rosetting Ly 1.5 times, whereas the murine DLL increased their number in average two times (Tab. 2). Although a principal difference in the capacity to restore SRBC receptors of human Ly by substances of human or murine origin was not observed, the human DLL seemed more appropriate when the number of cells (from which the amount of DLL was prepared) is calculated per one Ly used in the rosetting test.

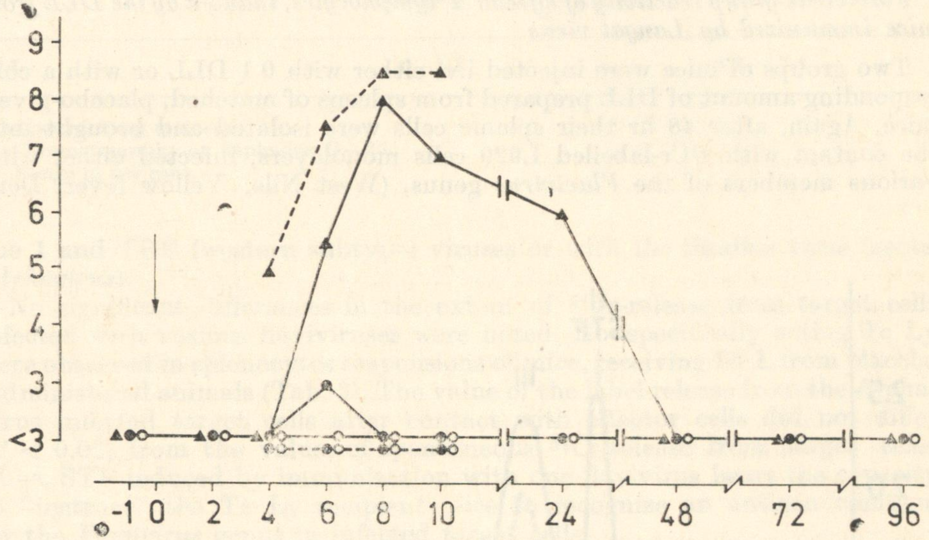

Fig. 3.
Induction of IFN by the attenuated Langat virus and by DLL from splenocytes of either immunized or normal mice
▲———▲ experiment 1; △------△ experiment 2; (▲) 10^3 PFU of Langat virus; (●) mice receiving DLL from immune splenocytes; (○) mice receiving DLL from control splenocytes. Abscissa: hr after inoculation; ordinate; lg_2 of IFN

Generation of the STF activity in splenic leukocytes

Cohort donor mice were immunized with the attenuated Langat virus. At daily intervals — until day 10 — always three mice were sacrificed and their pooled splenocytes stored at —18° C. After processing, the individ ual lysates were dialyzed, freeze-dried and reconstituted at use to obtain one DLL unit per ml. Always three recipient C3H mice were inoculated i. p., their splenocytes were used as effector cells to assess the STF activity of the lysates. First increment in the STF activity was observed in splenocytes of mice, sacrificed on 2nd or 3rd immunization days. At subsequent intervals a continuous increase of the STF production was observed (Fig. 1).

Time-dependence of STF activity expression in vivo

C3H cohort mice received i.v. 0.1 DLL units comprising 10^4 STF units. At intervals (Fig. 2) always three animals were sacrificed and their pooled splenocytes assayed for specific Tc cells. The first significant difference in the ^{51}Cr-release was stated as early as 15-21 hr after the DLL administration. The effect of the DLL increased further as witnessed by the percentage of the Tc cells causing ^{51}Cr-release at later intervals.

Flavivirus-group reactivity of effector T lymphocytes, induced by the DLL from mice immunized by Langat virus

Two groups of mice were injected i.v. either with 0.1 DLL or with a corresponding amount of DLL prepared from spleens of matched, placebo-given mice. Again, after 48 hr their splenic cells were isolated and brought into the contact with ^{51}Cr-labelled L929 cells monolayers, infected either with various members of the *Flavivirus* genus, (West Nile, Yellow fever, Den-

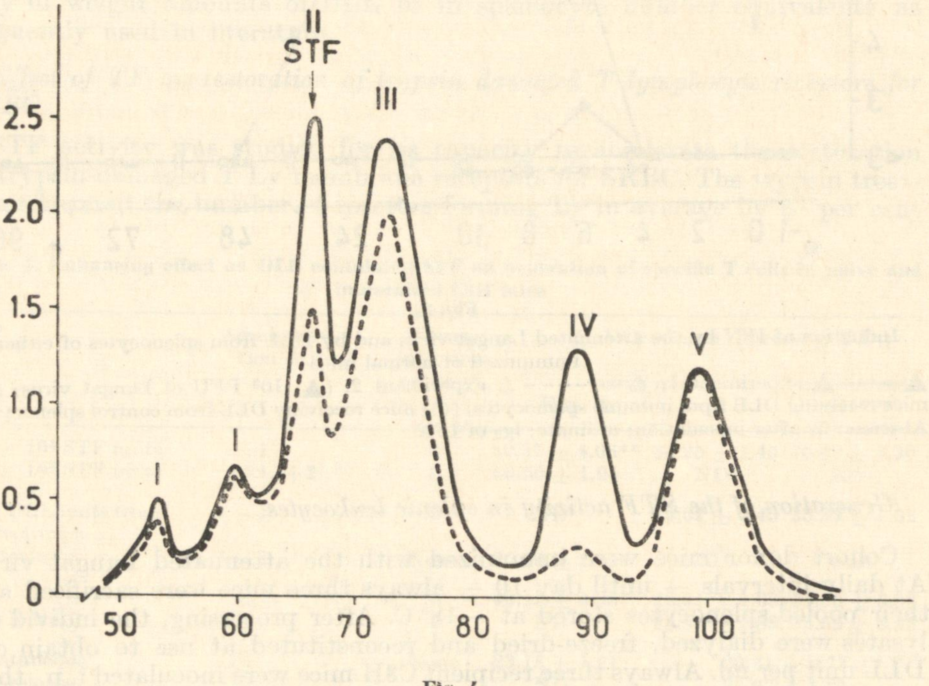

Fig. 4.

Elution profile of the DLL on Sephadex G-25 fine
Absorbance recorded at 260 nm (———) and 280 nm (— — —). Fractions (I, II, III, IV, V), were freeze-dried. SPF activity (arrow) was detected in ^{51}Cr release assay.
Abscissa: tube number; ordinate: relative optical density

Table 5. Ribose-bound (orcinol-reactive) material (ORM) and peptide contents in various batches of DLL and their fractions

Fraction*	ORM	Peptide
I.	0.3 – 0.5**	2.2 – 3.9
II.	0.8 – 2.8	7.2 – 10.6
III.	1.3 – 2.8	15.6 – 22.2
IV.	0.3 – 0.4	3.3 – 4.2
V.	0.3 – 0.6	2.8 – 3.9
DLL	1.0 – 1.3	6.3 – 7.4

Analysis of 2 mg material.
* Chromatography on Sephadex G – 25.
** Range in per cent.

gue 1 and TBE (western subtype) viruses or with the Sindbis virus (genus *Alphavirus*).

No significant differences in the extent of ^{51}Cr-release from target cells infected with various flaviviruses were noted. No specifically acting Tc Ly were observed in splenocytes suspensions of mice, receiving DLL from placebo -administered animals (Tab. 3). The value of the label release from the Alphavirus infected target cells after contact with effector cells did not differ ($P < 0.02$) from the values of spontaneous ^{51}Cr-release from target cells. Thus, STF induced by immunization with one flavivirus bears the capacity to "instruct" the Tc Ly recipient mice to recognize an antigen common for the *Flavivirus* genus in infected target cells.

Enhancing effect of STF activity on the killer T lymphocytes

In order to study the effect of DLL on generation of specific Tc cells in either non-primed C3H mice or in immunized and challenged ones, two experiments were designed. In the first approach (two parallel, but mutually idependent investigations), DLL was given prior the immunizing virus dose to recipient mice. In both series, the used amount of STF increased the in vivo response to virus by 59 – 60% when measured on the 3rd day after inoculation and compared to the response elicited by the virus only. In the second approach, cohort mice on the 11th day after immunization received a dose of 2.5×10^4 STF units (Tab. 4) and on the subsequent day they were challenged with the 10^5 PFU of the homologous virus. In mice, receiving the challenging virus alone, the Tc cells response was by 28 per cent lower than in STF recipients.

Interferon induction by the DLL

To investigate the effect of DLL administration on IFN induction, cohort mice were injected i.v. either with a single dose of 0.1 units DLL (10^4 STF

Table 6. Biological activity, orcinol-reactive material and peptide contents in the DLL and its fractions

	DLL*	Fractions I.	II.	III.	IV.	V.
ORM	17.4**	9	50	50	6	10
Peptide	114	40	130	280	60	70
STF (units)	10^4	1	10^4	ND	1	1
E-rosettes***	ND	1.7	14.9	9.1	9.0	−7.8

* Five units of DLL; 174 μg ORM and 1140 μg of peptides per unit.
** μg/2 mg of lyophilized material.
*** Per cent in the presence of DLL − per cent in the absence of DLL.
ND = not done.

units), or with a single dose of 0.1 units DLL prepared from non-immunized mice, or with 10^4 PFU of Langat virus. At intervals (Fig. 3), three C3H mice were bled, their sera pooled and investigated for IFN levels. After the administration of DLL from immunized mice, IFN was detected in a low titre at a single 6 hr interval only. The DLLs from non-immunized mice did not elicite a detectable IFN response. On the other hand, virus infection elicited a marked IFN response, starting from 6th hr attaining the peak level at 8th hr decreasing slowly, until 48 hr p.i.

Thermal inactivation of the STF activity

DLL containing 10^4 STF units per ml was dispended in 0.5 ml amounts in thin-wall rubber-stoppered test tubes. They were immersed under continuous shaking in a water bath (ultrathermostat temperature control ± ± 0.01 °C). At intervals, individual test tubes were removed, immediately cooled and stored frozen at −18° C until tested by the ^{51}Cr-release test. The temperature 37° C lowered the STF titre by 3 $\log_{10}$ units during 24 hr; no activity was detected in samples after 42 hr. During 60 min at 56 °C the STF activity was completely abolished, as measured by the Tc cell inducing capacity of the DLL sample.

Fractionation of DLL by gel filtration

The chromatography on Sephadex G-25 fine columns (dawnwards flow) of individual murine DLL batches, whether prepared from live virus-immunized or from placebo-given mice, resulted usually in separation of five fractions (Fig. 4). As hypothetical substrates of TF activity, protein and ribonucleotides (purine-bound ribose, ORM contents) were estimated in 2 mg of freeze dried DLL and in individual fractions. The proportionality calculations of values, obtained from individual materials showed that although the contents of these materials ranged relatively broadly (various batches of DLL), ribose and peptides were invariably more concentrated in the IInd and IIIrd fractions (Tab. 5).

The comparison of weight proportionalities in individual fractions showed — in each from numerous estimations performed — that almost 80—85 per cent of the investigated material is eluted in the Ist fraction. The IIIrd and Vth fractions contained lowest amounts of eluted materials (1.7—3.4%). For gel filtration experiments on the 2.5 × 90 cm columns using 3.25—5.2 DLL units, the material eluted comprised 42.7—56% of the initial weight of the material. No apparent difference in the ORM and peptide contents were noted between the DLLs and their fractions, whether prepared from immunized or placebo given mice.

The individual DLL chromatographic fractions, reconstituted after lyophilization, were studied for the presence of the STF activity and the ability to restore the SRBC-rosetting activity of the trypsin-treated human T Ly. In fractions No. I, III, IV and V, STF activity was never recovered, this remained always confined to the fraction No. II. In two representative experiments the quantitative relations between the STF activity in DLL and its fractions were analysed. For gel filtration 5.2 and 3.25 DLL units were used. i.e. 112 and 65 mg of the freeze-dried DLL, always reconstituted in one ml of bidistilled water. One mg of both starting materials contained 5×10^3 STF units. In one mg of the IInd fraction, 2.5×10^4 STF units were recovered in the first and 5×10^4 STF units in the second experiment. Although the total STF activity (expressed in STF units) recovered in the IInd fractions were 5.6 and 3.25 times lower than the total starting STF activity, its titres witnessed a relative concentration of the substrate. Consistently with the presence of STF activity in the IInd chromatographic raction, the most pronounced restorative capacity on trypsin-digested SRBC receptors of human T Ly was observed this material (Tab. 6).

As described in the section dealing with the DLL preparation, the majority of ORM (i.e. about 68%) and of the protein (i.e. about 64%) crossed the dialysis tubing during the second interval of dialysis (dialysate B). On the other hand, 99—99.9% of the STF activity was present in the dialysate A, containing 30—34% of ORM and proteins.

The lyophilized material, recovered during the second interval of the dialysis was chromatographed on the Sephadex G-25 column (Valášková *et al.*, in preparation). No STF activity was detected in lyophilized fractions of four peaks obtained.

Discussion

The murine model for STF investigation allows for the first time a quantitative exploration of antigen-dependent TF activity by estimating the generation of specifically "instructed" Tc cells. The system is suitable to the study of CMI response also in other than flavivirus infections. It has been repeatedly shown (for review see Khan *et al.*, 1979) that individual preparations or batches of DLL, not seldom referred to as TF, varied considerably in biological effectivity and biochemical composition, whether of human or animal origin. Our findings with the murine DLL are consistent with this observation. DLL and/or TF activities have been measured according to the number of lyzed leukocytes of Ly (usually 10^8-10^9 cells) used for obtaining one arbitrary unit of the product presumed to contain TF activity. In the murine model system used, one DLL unit (20 mg of the freeze dried DLL) corresponded to the dialysable material from 3×10^8 splenocytes. Such a quantity may be useful for to study various antigen-dependent and independent effects of the dialysate known to contain different low-molecular substances. To express STF activity exquisitely, we have introduced the STF units. In the TBE virus infected mice they were defined according to the capacity of DLL to generate Tc cells.

The biochemical study of the TF-containing materials up to now did not disclose the structure and/or composition of TF. It has been suggested (for review see Petersen and Kirkpatrick, 1979) that both, peptide and nucleotide are required to transfer the antigen-specific response. Actually, elution of these substances from Sephadex G-25 columns in relatively high amounts paralelled the presence of the STF activity in the second fraction. Accordingly, we investigated the purine bound ribose content and the protein content in all DLL fractions. Nonetheless, the relations between these values (especially in the IInd and in the IIIrd fractions) and the specific Tc cell generation capacity did not suggest a simple weight-effect relationship, showing the need of further biochemical analyses. Data from the study of the materials obtained by divided dialysis (Fig. 5) seem to support this view.

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