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ANTIGEN-SPECIFIC TRANSFER FACTOR FROM MICE IMMUNIZED WITH AN ATTENUATED FLAVIVIRUS: AUGMENTATION OF INDUCING ACTIVITY IN SEMIPURIFIED SPLENOCYTIC DIALYZATES

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Summary. — Three large batches were prepared of lyzed splenocytic leukocyte dialyzate from SPF outbred mice, immunized with a live attenuated virus from the tick-borne encephalitis (TBE) complex. Total mass of freeze-dried dialyzates was 1.73 g. One mg of respective batches contained 2×10^5, 2×10^4 and 2×10^3 units of the transfer factor, specific for the flavivirus group-antigen, as estimated according to the capacity to induce specifically cytotoxic T-cells in the recipient C3H mice. The amount of protein and orcinol-reactive material (purine-bound ribose), the presumed components of the inducer's substrate, ranged in individual dialyzates from 9.9—12.4 and 0.72—0.80% of their dry mass. Materials from each batch obtained after double precipitation by ethanol were subjected to permeation chromatography on Sephadex G-25 columns and subsequent lyophilization of the peak with specific inducing activity. The final product represented on average 3.7 per cent of dry mass of the starting material. In comparison to the crude material, in one mg of the final product the protein and the orcinol-reactive material were reduced by 80 and 37 per cent, respectively, but an increment in the antigen-specific inducing capacity comprising 2—3 $\log_{10}$ units was observed. These findings add to the concept that a) macromolecules carrying the inducing activity can be separated from other constituents of the crude dialyzate and b) an increase in antigen-specific inducing activity titre was, besides partial concentration, mainly due to removal of suppressor or inhibitory factor(s) present in the crude dialysates and probably acting in vivo.

Key words: transfer factor; flavivirus; antigen-specificity; purification, supressor factor

Introduction

The dialyzable >3 500—<12 000 Da antigen-specific moiety, residing in helper T-lymphocytes (T-ly) of immune donors, inducing the cell-mediated immune response (CMI) upon injection into a non-primed immunocompetent recipient is designated as transfer factor (TF) (for review see Lawrence and Borkowski, 1983). It appears that TF acts via a shunt that bypasses the stage of active immunization and converts naive T-ly to an antigen--responsive state. Once this event takes place, being a matter of hours, the recipient's T-ly acquire the in vivo and in vitro capacities of cells which result from accomplished active immunization of the given specificity. In the case of viral infections, this conversion was demonstrated in studies with TF obtained after experimental infection with a live flavivirus (Mayer *et al.*, 1980, 1982).

The interest in this particular mode of "immunological engineering" resulted in a number of remarkable analytic and biological studies as well as in a number of clinical ("phase I" trials) observations (for review see e.g. Kirkpatrick *et al.*, 1983). Nevertheless, other similarly conducted studies were not conclusive, because the inducing effectiveness of the dialyzates used varied greatly, what was ascribed to an unpredictable heterogeneity in their composition. Moreover, the lack of suitable experimental animal models and of a reproducible in vitro assay hampered, until recently, to outline more clearly expressed working conceptions.

The crude dialyzate of lyzed leukocytes (DLL), being the only useful source of TF, represents a complex mixture of cell-liberated low-molecular weight materials (e.g. Hamblin, 1979; Nekám *et al.*, 1981). As recently shown (Holzman *et al.*, 1983) the crude DLLs, prepared from the whole leukocytic population of healthy human donors constantly contains in varing amounts substances exerting nonspecific inhibitory and specific suppressoric effects on the TF interactions with the cells of the immune system. Suppressoric activity does not only block the TF's inducing activity, but abrogates also the response of already immune cells to the specific antigen (Lawrence and Borkowski, 1983). In order to improve the efficiency of TF preparations, the removal or at least a significant decreases of the suppressor factor content (designed also as "anti-TF") from DLL would appear desirable (Chase, 1983).

This report extends our recent attempts — also working with a model murine system — to increase the antigen-specific TF activity by a combination of ethanol precipitation of DLL and of gel filtration (Mayer *et al.*, 1983*b*). Evidence is presented — in terms of protein and of purine-bound ribose (orcinol-reactive material, ORM) and removal of "non-TF" substances — about the composition of such a semipurified final product, together with its inducing activity levels, quantitated according the potency to generate antigen-specific cytotoxic T cells (T_{cx} cells) in vivo (Mayer *et al.*, 1980, 1983*a*).

Materials and Methods

Viruses. 1. The E5"14" clone of the Langat virus, belonging to the tick-borne encephalitis (TBE) complex, avirulent for subcutaneously inoculated subadult SPF mice, was grown in brains

of intracerebrally (ic.) inoculated newborn mice. One ml of the virus material in the form of ten per cent brain suspension (stored as a large stock in aliquots at —70 °C) contained 8.07×10^7 PFU on PS cell (pig kidney epithelial cell line) monolayers (Mayer and Kožuch, 1975) and 8.7 log i.c. LD_{50} for subadult SPF mice. 2. The "204" strain of the TBE (western subtype), obtained from the WHO Collaborating Centre for Arboviruses, Institute of Virology, Slovak Academy of Sciences, Bratislava, which underwent 7 brain passages in subadult SPF mice in this laboratory, One ml of the stock virus material, stored in the form of ten per cent mouse brain suspension, titered 8.1×10^7 PFU on PS cell monolayers, 9.6 log i.c. LD_{50} and 8.0 log subcutaneous (s.c.) LD_{50} in subadult SPF mice.

Mice. Cohorts of 200 — 400 outbred SPF subadult mice (strain "ICR"), were used as DLL donors after s.c. administration of a single dose of 10^4 PFU of low virulent Langat virus. Groups of matched control mice, inoculated with appropriately diluted ten per cent non-infected mouse brain suspension were used for preparation of DLL without flavivirus-specific TF activity. The "ICR" mice were used also for infectivity titrations of the TBE viruses suspensions. As TF recipients, inbred male 4 — 5 weeks old C3H/Cbi/BOM/H-2^k mice were used from the "Šumice" breeding farm.

Preparation of lyzed murine splenocyte dialyzates. 3 — 5 weeks after the administration of live virus or placebo, the splenic leukocytes of donor mice were harvested from finely minced spleens by mechanical coarsing. One spleen yielded cca 10^8 leukocytes under conditions of larger scale preparation. After osmotic lysis of erythrocytes, present in the cell suspension, the repeatedly washed splenocytes were disintegrated by ten cycles of freezing and thawing (CO_2-alcohol bath and 37 °C warm water). The resulting material, with added DNase and $MgSO_4$, was then dialyzed for 48 hr/4 °C against 20 vol of sterile bidistilled water. The dialyzate was shell-frozen and freeze-dried. The DLL from placebo administered mice was prepared in a similar way. Individual batches of DLL represented pooled yields from 400 — 600 spleens. The lyophilized substance was stored at —20 °C in tightly stoppered vials.

Titration of the antigen-specific TF inducing capacity. The capacity of the DLL and of other materials derived from it and containing the antigen-specific TF activity to induce T_{cx} cells, recognizing the TBE virus neoantigens, was assayed in recipient inbred C3H mice. They were inoculated intraperitoneally with 0.25 ml material studied, dissolved in sterile bidistilled water and serially (log_{10}) diluted. By 48 hr after inoculation, spleens of the recipient mice were aseptically removed, suspensions of washed leukocytes prepared and inoculated (50 : 1) into test tube cultures of syngeneic L-929 cells. Cell cultures were previously infected with TBE virus strain "204" and labelled with ^{51}Cr as described (Gajdošová *et al.*, 1980; Mayer *et al.*, 1982). The antigen-specific TF activity endpoint was calculated (log_{10} or antilog values) from the highest dilution of the given material inducing the generation of specific T_{cx} cells in the spleens of recipient mice. The percentage of the release of ^{51}Cr from target cells at the end point of TF activity differed significantly ($P < 0.001$) from the percentage of the spontaneous ^{51}Cr release in TBE virus-infected and in uninfected cells, as well as in target cells inoculated with splenocytes from placebo-administered donor mice. The titre of this antigen-specific TF activity (TF units, Mayer *et al.*, 1982) was calculated always for one mg of the dried material investigated.

The partial purification method, based on double precipitation of the DLL lyophilizate by ethanol with subsequent chromatographic separation, was described in details in the preceding paper (Mayer *et al.*, 1983*b*). Shortly, to the given amount of the dissolved DLL, two volumes of chilled 96 °C ethanol were added. The fine precipitate (P 1), formed after one hour at 4 °C was removed by centrifugation. To the supernate (S 1) further two volumes of ethanol were added. After 4 hr at 4 °C, the second precipitate (P2) was harvested by centrifugation at 900 g/20 min. The P1 and P2 materials, well dissolving in bidistilled water, were freeze-dried and stored at —20 °C until further processed. Starting DLLs, P1 and P2 materials were then dissolved in water and subjected to the gel permeation chromatography on 1×50 cm Sephadex G — 25 (fine) column, with bidistilled water as eluent, at an effluent rate of 11 ml per hour.

Descending flow was used. Two ml samples were collected and monitored for extinction values at 260 and 280 nm. From individual tubes appropriate pools were prepared. From the pools, resulting from the P1 and P2 separation. the fractions containing the antigen-specific TF activity and designed as PL/II and P2/II were used for further analyses, always after being freeze-dried.

Determination of protein and of purine-bound ribose. The amount of protein in materials investigated was determined by the method of Lowry *et al.* (1951), the purine-bound ribose by the method of Mejbaum (1939).

Results

Starting dialyzates of lysed murine splenocytes

We used three batches of DLL, prepared from mouse splenocytes by a standard method of single-shot immunization with live Langat virus. The batches, designed "B", "C", "D", represented 595, 333 and 800 mg of the freeze-dried substance. The individual batches were deliberately selected in order to contain a relatively high level of the antigen-specific TF activity ("B", 2×10^5 PF units per mg), or titering lower by one and by two $\log_{10}$ orders ("C", 2×10^4 TF units per mg; "D", 2×10^3 TF units per mg). Chromatography of these materials on Sephadex-G 25 column yielded similar elution profiles, the second peak being the highest and the only one from which the antigen-specific TF activity was recovered.

The protein and ORM contents comprised on average 11.6% (range 10.6—13.2%) of the total mass of DLLs examined. Thus, unlike the specific activity, these amount did not markedly differ from individual starting materials, whether calculated for the total mass of batches studied or per one mg of them (Tables 1 and 2). On the other hand, this means that on average 88% of the starting materials was of another chemical nature.

Partial purification of the antigen-specific TF activity carrier substrate present in DLL

The individual purification steps are characterized in terme of protein and ORM contents as well as by the amounts of non-protein/ORM material.

For further processing usually the S1, but not the P1 precipitate was used. Nevertheless, the P1 material represented 2—14.7% of the amount of starting DDL. The lyophilized P1 materials contained only 2.7—7.6% of protein and 0.35—0.93% of ORM. From the average P1 mass of 31 mg (range 16—49 mg), the above substances comprised on average 5.8%. The average mass of the protein/ORM components of P1 materials was 1.73 mg, i.e. about 94% of the first ethanol DLL precipitate was of another nature.

From the total TF activity in individual starting batches, the total antigen-specific TF activity in P1 materials represented 0.47% in "B", P1, 14.8% in "C" P1 and 2% in "D" P1 (Table 1). If the TF activity titre per one mg of each P1 and one mg of the respective starting DLL was calculated, then in the case of the "C" and "D" materials, no differences in this parameter were observed, with an exception of the "B", where one $\log_{10}$ decrease was found.

Interestingly, when from the "B" P1 precipitate the fraction containing TF activity (i.e. P1/II) was chromatographically separated, it constituted 42.8% of its mass. Protein content per one mg was lower by 67.5 and 76% in P1 and "B" DLL, respectively. Similarly, ORM content was lower by 50% in P1 and by 37.5% in "B" DLL. Nevertheless, 97% of the freeze-dried P1/II material was of non-protein/ORM character (Table 1). In spite of the decreased protein/ORM contents, the antigen-specific TF activity was 10-fold higher in the P1/II than in P1, thus equalling to the titre in "B" DLL.

Table 1. Partial purification of dialyzates of splenic cells from live flavivirus-immunized mice, expressed in terms of protein, orcinol-reactive material (ORM) and of antigen-specific TF inducing activity contents

Material	Batch	Mass of lyophilized substance		Protein		ORM		TF titre of the total mg	Ratio of TF titres in the given and starting material
		mg	%	mg	%	mg	%		
DLL	B	595	100	61.88	10.40	4.28	0.72	1.19×10^8	
	C	333	100	32.96	9.89	2.44	0.73	6.6×10^6	
	D	800	100	99.20	12.40	6.40	0.80	1.6×10^6	
P 1	B	28	4.70	2.15	7.67	0.10	0.35	5.6×10^5	0.005
	C	49	14.71	1.86	3.79	0.25	0.51	9.8×10^5	0.14
	D	16	2.00	0.68	4.25	0.15	0.93	3.2×10^4	0.02
P 1/II	B	12	2.01	0.30	2.50	0.05	0.45	2.4×10^6	0.02
P 2	B	37.6	6.21	2.40	6.38	0.19	0.50	7.52×10^7	0.63
	C	53.0	15.91	1.11	2.09	0.26	0.49	1.06×10^8	16.06
	D	55.0	6.87	2.20	4.00	0.20	0.36	1.1×10^7	6.87
P 2/II	B	23	3.86	0.46	2.00	0.13	0.56	4.6×10^8	3.86
	C	18	5.40	0.24	1.33	0.07	0.38	3.6×10^8	54.50
	D	23	2.87	0.78	3.39	0.08	0.34	4.6×10^7	28.75

Symbols of materials from individual steps of purification by gel filtration of alcoholic precipitates of dialyzates of splenic leukocytic cells (DLL) are explained in "Materials and Methods"
ORM: purine-bound ribose
TF antigen-specific activity measured according generation of cytotoxic T cells in ^{51}Cr— release assay (TBE virus-infected target L929 cells)

Table 2. Protein, orcinol-reactive material (ORM) and antigen-specific TF activity in one mg of materials from individual purification steps of splenocytic leukocyte dialysates from flavivirus-immunized mice

Material	Batch	Protein		ORM		TF units	Concentration factor
		µg	%	µg	%		
DLL	B	104	100	7.2	100	2×10^5	
	C	99	100	7.3	100	2×10^4	
	D	124	100	8	100	2×10^3	
	means	**109**		**7.5**			
P 1	B	77	74.03	3.6	50	2×10^4	0.1
	C	38	38.38	5.2	71.23	2×10^4	0
	D	43	34.67	7.2	90	2×10^3	0
	means	**52.66**	**49.02**	**5.3**	**70.41**		
P 1/II	B	25	24.03	4.5	62.5	2×10^5	0
P 2	B	64	61.53	5.2	72.22	2×10^6	10
	C	21	21.21	5	68.49	2×10^6	100
	D	40	32.25	3.8	47.50	2×10^5	100
	means	**41.66**	**38.39**	**4.66**	**62.73**		
P 2/II	B	20	19.23	6	83.33	2×10^7	100
	C	13.5	13.63	4.3	58.90	2×10^7	1000
	D	34	27.41	3.8	47.50	2×10^6	1000
	means	**22.5**	**20.09**	**4.7**	**63.24**		

For explanations see Table 1

This titre increment suggests a decrease in an „anti-TF" activity due to gel filtration rather than solely to the TF substrate concentration.

The P2 precipitates dry weight, ranging from 37.6—55 mg (average 48.5 mg), comprised 6.31—15.9% (9.7% on average) of the initial mass of the DLL batches studied (Table 1). The protein and ORM contents, calculated per one mg of the substance, were on average lower in P2 than in P1 materials (Table 2), both constituting 2.6—6.9% (average 4.7%) of the P2 materials dry mass. This means also that 93—97% of the dry P2 substance was of a non-protein/ORM character.

The recovery of the total antigen-specific TF activity in the "B" P2 (i.e. in 37.6 mg of substance) was 63.2% of the total activity in the crude "B" DLL, although the weight of the P2 precipitate represented only 6.31% of its amount. In the case of the "C" P2, the total TF activity recovery was 16.6-fold and in the case of "D" P2 6.9-fold higher than that of the respective starting crude DLL amounts. While the mass of P2 materials was on average 48.5 mg, that of the P2/II chromatographic fractions decreased to 21.3 mg (range 18—23 mg), constituting only about 3.7% (range 2.9—5.4%) of the starting crude DLL dry mass. The protein content in the P2/II dry materials ranged from 1.3—3.4%. The ORM content represented in these materials 0.34—0.56%. The average proportion of the protein/ORM in the dry P2/II fractions was 2.7, thus again, more than 97% of this finally obtained substance displayed another chemical nature.

The total protein amount in the crude DLLs was 194 mg, the ORM total amount 13.2 mg. These values for the P2/II materials were 1.48 and 0.28 mg (Table 1). In other words, the combined purification procedure removed 99.2% of protein and 97.9% of ORM, originally present in the crude DDL. The total non-protein/ORM material weighed in the starting DDL 1521 mg, i.e. it represented 88%. For the P2/II total material this non-protein/ORM amount was 62.2 mg, i.e. about 97% of the final product mass. Nevertheless, this amount comprised only 4% of the total starting non-protein/ORM material.

When the results of the analyses were calculated per one mg of the given materials, then P2/II had been devoid on average of 80% of protein and of 37% ORM by the purification procedure (Table 2). Study of the TF capacity of the P2/II materials showed that in spite of minute amounts of protein and ORM materials, the total amount of TF inducing activity, expressed in TF units, increased 3.8—54.5 fold (Table 1), as compared to the starting values detected in individual DLL. In terms of TF inducing capacity calculated per one mg of materials assayed, this antigen-specific activity increased in the P2/II final products 100—1000 fold (Table 2).

Discussion

Among the numerous low-molecular weight components in the crude DLL, exerting various biological activities, TF is unique in its ability to combine in immunologically specific manner with the specific antigen either in vitro

and/or in vivo to confer in the non-primed recipient an antigen-specific CMI response. Its antigen-combining capacity may be due to a V_H region determinant expression in TF structure (Borkowski and Lawrence, 1983), confined probably to its peptide component (Kirkpatrick *et al.*, 1983). The present evidence is suggestive of oligoribonucleopeptide nature of the TF (Paddock *et al.*, 1983).

Further, the crude DLL was found to contain many immunopharmacologically active substances with nonspecific adjuvant or suppressor functions (Holzman *et al.*, 1983; Chase, 1983). Some of these "non-TF" materials, e.g. the alpha$_1$-thymosin (Wilson *et al.*, 1983), even augment subnormal proliferative or inductive responses to the antigen. Assuming that protein and ORM substances participate in the TF's substrate, then the "non-TF" substances in DLL may constitute, as shown in present experiments, not less than 88% of the crude DLL's mass and 97% of the mass of the semipurified material studied. It seems acceptable that the "non-TF" material composition may not be the same one in the crude and in the final semipurified product. The character of these "non-TF" materials, however, has not been analysed as yet concerning their interactions with TF.

Recent developments in the field have shown that one and the same preparation of DLL contains at least two antigen-specific, but functionally opposing activities of the same molecular weight class (Borkowski and Lawrence, 1983; Fudenberg *et al.*, 1983; Holzman *et al.*, 1983; Kirkpatrick *et al.*, 1983; Lawrence and Borkowski, 1983). The first induces the CMI manifestations in the recipient, i.e. exerts the TF activity and the other one, suppresses the effect of the former. Moreover, the 3 500 Da dialysis fraction contains besides an array of substances with non-specific adjuvant effects also constituents inhibiting without an antigenic specificity, the inductive activity of the TF (Lawrence and Borkowski, 1983). The specific suppressoric activity, the "anti-TF", is most probably responsible for the irregularities and differences in the inducing activities observed between individual crude DLL batches. Proportionality, at least between the regulatory substances mentioned, favorizing the beneficial TF's effect is difficult to predict for individual DLL preparations of unpurified leukocytes of unselected donors (Holzman *et al.*, 1983). Extremely laborious investigative approach to the problem of more regularly successful TF immunomodulation effect was advised by Fudenberg *et al.* (1983), showing the unusual complexity of a rational application of the crude DLL.

A very marked increase in the antigen-specific TF inducing capacity as measured by generation of T_{cx} cells in previously non-primed C3H mice was observed in the materials obtained after exclusion chromatography of alcoholic precipitates of DLL from mice immunized with low-virulent virus. This, together with protein and ORM analyses, implies the following. Per one μg of the protein/ORM material in the crude DLL we detected 1799 TF units in the case of the "B", 188 TF units in the "C" and 15 TF units in the case of the material "D". In the final product, however, per one μg of the protein) ORM material we detected 7.7×10^5 TF units in the case of the

material "B", 1.12×10^6 TF units in "C" and 5.3×10^4 TF units in the "D" material. This means that when the purification procedure removed 94.6 — 97.1 % of the crude dry mass (Table 1); although the resulting product contained only 20 % of the original protein amount and only 63 % of the original ORM amount — per one mg of the substance recovered — it appears that the final materials were free of an activity exerting a suppressor effect. Thus the latter activity seems to differ from the oligoribonucleopeptide nature of TF. Our findings indicate that for obtaining relatively pure, highly specific and potent materials for induction or augmentation of CMI (T_{cx} cells), the semipurification approach may be useful. This fact may be not without significance for clinical testing of TF preparations, where a more standard potency of deliberately selected TF antigen-specificity is required.

It cannot be absolutely excluded, however, that TF has another structure for which the protein/ORM moiety is not essential and that TF was simply copurified with it. Such a possibility is not consistent with the results of others (e. g. Burger *et al.*, 1983) on TF structure and would contradict to the prevailing consent. In the crude DLL, as can be judged according to the titration of as yet 30 different batches of TF from live virus-immunized mice, TF activity never attained such high values as observed in semipurified materials, as compared to the starting crude DLL. From these results that if the TF activity (induction of T_{cx} cells, possibly also stimulation of NK cells and of cytotoxicity-exerting macrophages) is calculated per one μg of the protein/ORM material, the semipurified product contained in the case of the "B" batch 426 ×, in the case of the "C" batch 5957 × and in the case of the "D" batch 3533 × more antigen-specific acitivity as the same amount of mentioned substances in the respective crude materials. This seems at best explained assuming that "C" and "D" showing in crude state considerably lower TF activity, contained also higher amounts of the suppressor activity than the material "B", showing the relatively highest TF titre already in the crude DLL form. Described situation may parallell the differences observed while using human DLL. Nevertheless. also various purification procedures should be investigated, in order to select the most efficient ones. Promising seems, e. g. the use of affinity chromatography on immobilized m-aminophenyl boronic acid, as described in a companion paper (Borvák and Mayer, 1985).

Actually, in recent experiments, from the crude murine DLL was separated a substance exerting antigen-specific suppressor effect on the inducing activity of TF, evoked in mice by administration of a live flavivirus. This suppressor activity present in chromatographic fraction of the DLL can be removed by the purification method described (Mayer *et al.* — in preparation).

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