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CONCENTRATION OF MURINE ANTIGEN-SPECIFIC TRANSFER FACTOR OF DEFINED POTENCY

V. MAYER, M. VALÁŠKOVÁ, E. GAJDOŠOVÁ, *C. ORAVEC

Institute of Virology, Slovak Academy of Sciences, 817 03 Bratislava; Joint Virus Research Centre, Clinic of Infectious and Parasitic diseases, Medical Faculty, J. A. Komenský University, Bratislava; and *Institute of Experimental Oncology, Slovak Academy of Sciences Bratislava, Czechoslovakia

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Summary. — Dialysates containing transfer factor (TF) activity were prepared from lyzed splenic cells of SPF mice immunized with live, peripherally avirulent Langat virus (TP21 E5 "14" clone) from the tick-borne encephalitis (TBE) complex. The amount of TF was estimated by its capacity to generate in recipient inbred C3H mice cytotoxic T lymphocytes inducing lysis of TBE virus-infected target cells as demonstrated by ^{51}Cr--release assay. A 100 to 1000-fold concentration of TF activity was achieved by combination of the two-step ethanol precipitation of crude dialysates with subsequent fractionation on Sephadex G-25 by exclusion chromatography. Materials from individual concentration steps showed reduced amounts of admixtures, as revealed by absorbance profiles of their chromatograms. In the final product the protein content was most decreased.

Key words: *transfer factor*; *antigen-specificity*; *concentration*; *tick-borne encephalitis virus*; *cytotoxic T lymphocytes*

Introduction

As established, TF influences the expression of the cell-mediated immunity transferring donor-related activities to the naive recipient (Lawrence, 1974).

Further progress in the investigations on nature and effects of TF still appears to be hampered by complex composition of its sole source, the leukocytic dialysate.

In unfractionated crude dialyzed leukocyte lysates (DLL) revealing TF activity, a wide variety of other biologically potent and immunologically active low molecular moieties was identified, e.g. prostaglandins and many others (Hamblin *et al.*, 1979). They may confuse, obviously, the results of various in vitro tests used to study relationships of the TF and cell--mediated immunity effector mechanisms (Gottlieb *et al.*, 1979; Peetom and Florey, 1979; Wilson *et al.*, 1979). It is also far not elucidated, whether the immunotherapeutic administration of such a mixture of heterogeneous

effector molecules enhances, or is adverse to the expected TF beneficial effects. In attempts to determine a rational for TF dosage, at least in some known clinical indications, and to monitor with a greater reliability the recipient organism response, some of the current trends are oriented towards the development and application of more purified and concentrated antigen--specific preparations of known potency. Considering these purposes we present the relatively efficient procedure of the TF activity concentration, removing simultaneously some admixtures from the mouse DLL preparations by combining the two-step ethanol precipitation (Klesius and Fudenberg, 1977) with chromatographic separation. As a model, the murine DLL was used; it contains TF activity specific for the *Flavivirus* genus antigen generated by immunization with an attenuated virus from the TBE complex (Mayer *et al.*, 1980, 1982).

Materials and Methods

Viruses. 1) The low virulent E5 "14" clone (Mayer, 1975) of the Langat TP 21 virus (TBE complex), reproduced in brains of intracerebrally (i.c.) inoculated newborn mice, was used for TF induction. One ml of the virus in the form of 10% brain suspension (stored at −70 °C) contained 8.1×10^7 PFU when plated on pig kidney epithelial cell monolayers (PS line) in plastic dishes and 8.7 dex i.c. LD_{50} for subadult SPF mice. 2) The "204"strain of TBE (western subtype) virus was obtained from the WHO Collaborating Centre for Arboviruses, Institute of Virology, Slovak Academy of Sciences, Bratislava. In this laboratory it underwent four passages in subadult mice brain. One ml of stock virus, stored in the form of 10% mouse brain suspension, had a titre of 6.4×10^6 PFU on PS cell monolayers, 8.7 dex i.c. LD_{50} and 6.5 dex subcutaneous LD_{50}.

Mice. SPF outbred mice, weighing 10−12 g intraperitoneally (i.p.) immunized with a single dose (10^4 PFU) of the peripherally avirulent Langat virus, were used for TF production. Matched groups of mice, administered 0.14 mol/l NaCl, were used to obtain the antigen-nonspecific DLL. The male inbred C3H/Cbi/BOM/H-2^k mice were used as TF recipients and subsequently as donors of cytotoxic T (T_{cx}) lymphocytes.

Preparation of dialysates of lyzed murine splenic leukocytes (DLL). The spleens of SPF outbred mice were removed 3 weeks after immunization and cell suspensions prepared from them by mechanical coarsing. After the treatment with 0.82% NH_4Cl, the washed splenocytes were resuspended in bidistilled water, counted and disrupted by freezing and thawing, repeated 10 times. The cell lysate was then dialyzed for 48 hr/4 °C against 20 volumes of bidistilled water The dialysate was shell-frozen and lyophilized. Individual lots represented pooled material obtained from 200−600 mice. The dry substance was stored at −30 °C. Twenty ml of this material were arbitrarily considered as one DLL unit (Mayer *et al.*, 1982).

Titration of TF antigen-specific activity. The capacity of TF to induce in recipient mice T_{cx} cells, recognizing the TBE virus neoantigens was assayed in inbred C3H, to which serial dilutions of individual materials tested were inoculated i.p. in 0.25 ml / mounts. After 48 hr, spleens were harvested, splenocyte suspensions were prepared and inoculated into test tubes with syngeneic L929 cell monolayers (50 ; 1) previously infected with the TBE virus (strain "204"), as described (Gajdošová *et al.*, 1980: Mayer *et al.*, 1980). The TF specific activity end-point was expressed in $\log_{10}$ values of the highest dilution of given material, inducing the generation of specific T_c cells in the spleens of recipient animals and causing ^{51}Cr-release of target cells. The percentage of this cytotoxicity differed significantly ($P < 0.001$) from the spontaneous ^{51}Cr-release in TBE--virus infected or non-infected target cells. T lymphocytes from recipient mice, injected with placebo were used as controls. The titre of the TF specific activity (TF units, Mayer *et al.*, 1982) was then expressed as neg $\log_{10}$ value of the end-point dilution (or its antilog value) and calculated for one ml of the undiluted material studied, containing usually 0.1 DLL unit or 2 mg of other dried materials.

Ethanol precipitation. The method advised by Klesius and Fudenberg (1977) was applied to the mouse DLL. The given amounts of DLL units were dissolved in 4—12 ml of sterile apyrogenic water in conical centrifuge vessels. Immediately thereafter, two volumes of the 96% ethanol were dropwise added. Fine precipitate was formed already during the ethanol addition. After gentle, but thorough mixing, the material was held for one hr at 4 °C. Precipitate, termed P 1 (Fig. 1) was removed after being spun down and further 2 volumes of ethanol were added to the supernatant (S 1). After 4 hr standing at 4 °C, the second precipitate (P 2) was harvested by centrifugation for 20 min at 900 × g at room temperature. The P 1 and P 2 materials, dissolving easily in bidistilled water, were freeze-dried, weighed and stored at −30 °C before estimation of their antigen-specific TF activity.

Gel chromatography. 5—7 DLE units were dissolved in water and fractionated on Sephadex G-25 (fine) columns (2.5 × 90 cm) as described (Mayer *et al.*, 1982), in order to characterize the starting material used for alcohol precipitation. The lyophilized P 1 and P 2 precipitates were dissolved in water and subjected to gel filtration in a 1 × 50 cm Sephadex G-25 column with bidistilled water as eluent, at an effluent rate of 11 ml per hr. Descending flow was used and tube volumes of 2 ml were collected. Extinction values at 260 and 280 nm were determined for in-

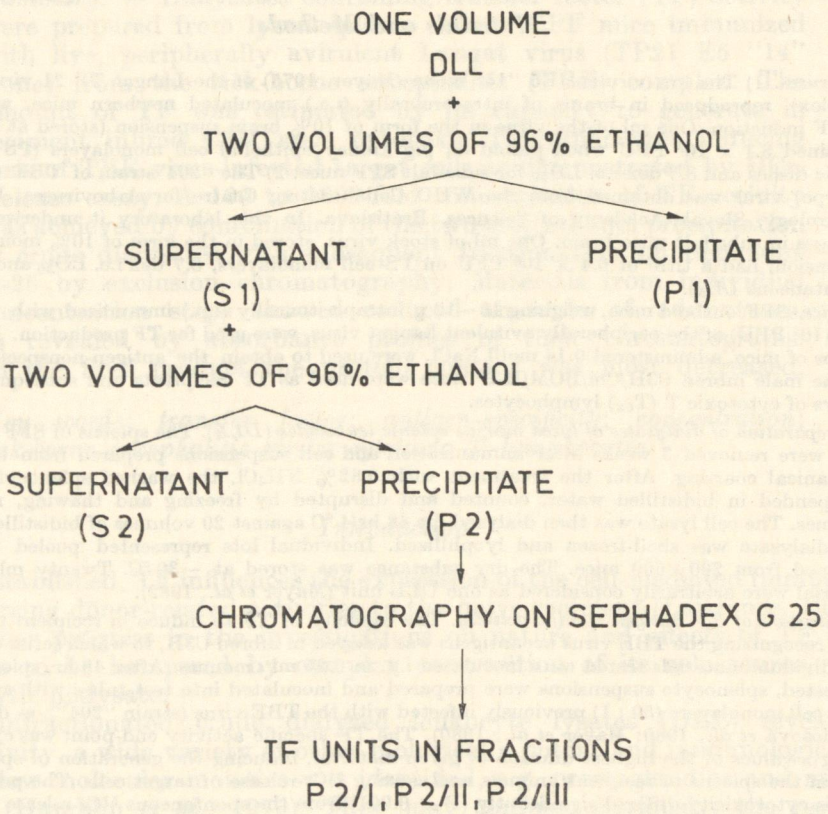

Fig. 1.
Procedures used for concentration of murine TF to the flavivirus group-specific antigen

Table 1. Concentration of flavivirus-specific TF activity by a combined ethanol precipitation and gel filtration

Material	Experiment	Protein (mg)		TF units	TF ratio **
Lyophilized DLL	A	200	(100%)	4×10^5	
	B	595	(100%)	1.19×10^8	
	A	2 ml*		8×10^3	0.02
	B	28	(4.7%)	5.6×10^5	0.005
P 1/II	A	4.7	(2.3%)	ND	
	B	12	(2.0%)	2.4×10^6	0.02
S 2	A	5 ml		2×10^2	0.0005
P 2	A	2 ml*		8×10^4	0.2
	B	37.6	(6.3%)	7.52×10^7	0.63
P 2/II	A	11.8	(5.9%)	2.36×10^7	59
	B	23	(3.8%)	4.6×10^8	3.86

* Precipitate dissolved in a given amount of distilled water
** Titre ratio of the given versus starting materials
DLL = Dialysate of lyzed splenocytes from mice single-shot immunized by the low-virulent Langat virus: A = lot DLL "10—15", 1 mg contained 2×10^3 TF units; B = lot DLL "19—21", 1 mg contained 2×10^5 TF units.
ND = not done
For further symbols see Fig. 1.

dividual samples, from which appropriate pools (fractions) were prepared. Individual fractions were freeze-dried and titrated for the T_{cx} cells inducing activity.

Determination of protein and of purine-bound ribose. The amounts 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) as orcinol-reactive material (ORM).

Results

Starting dialysates of mouse splenocyte extracts

For reported concentration attempts, large lots collected from several batches of DLL were used. They were designated „10—15" (1436 mg, used in exeriments A, C) and "19—21" (979 mg, used in experiment B). The specific cytotoxic T cells -inducing capacity of these materials represented 4×10^3 and 4×10^5 TF units per 2 mg of freeze-dried preparations (Table 1). Sephadex G-25 column eluates of these DLL materials showed the TF activity confined invariably to the fraction, constituting the second peak in the absorbance profiles of samples investigated (Fig. 2).

Concentration of TF activity

For experiment A, an amount of 10 DLL units (200 mg), for experiment B—27.75 units (595 mg) and for experiment C—5 DLL units (100 mg) of the

crude lyophilizate were used. The applied approach to concentrate the TF activity from the crude DLL is schematically outlined in Fig. 1. This method yielded final products with increased levels of TF activity. At the same time, the P2/II fraction was devoid of about 80% of protein (per 1 mg of the freeze-dried substance), providing that the initial value of protein content was 104 μg/mg of DLL (experiment B).

TF activity of the P 1 total precipitate, collected after one hr at 4 °C from the water-dissolved DLL and ethanol mixture (1 : 2) represented in the experiment A 0.4 % and in the experiment B 2 %, C respectively when compared to the total specific starting DLL activity. On the other hand, from the P2 precipitate (Table 1) as many as 20, 63.2, and 20 per

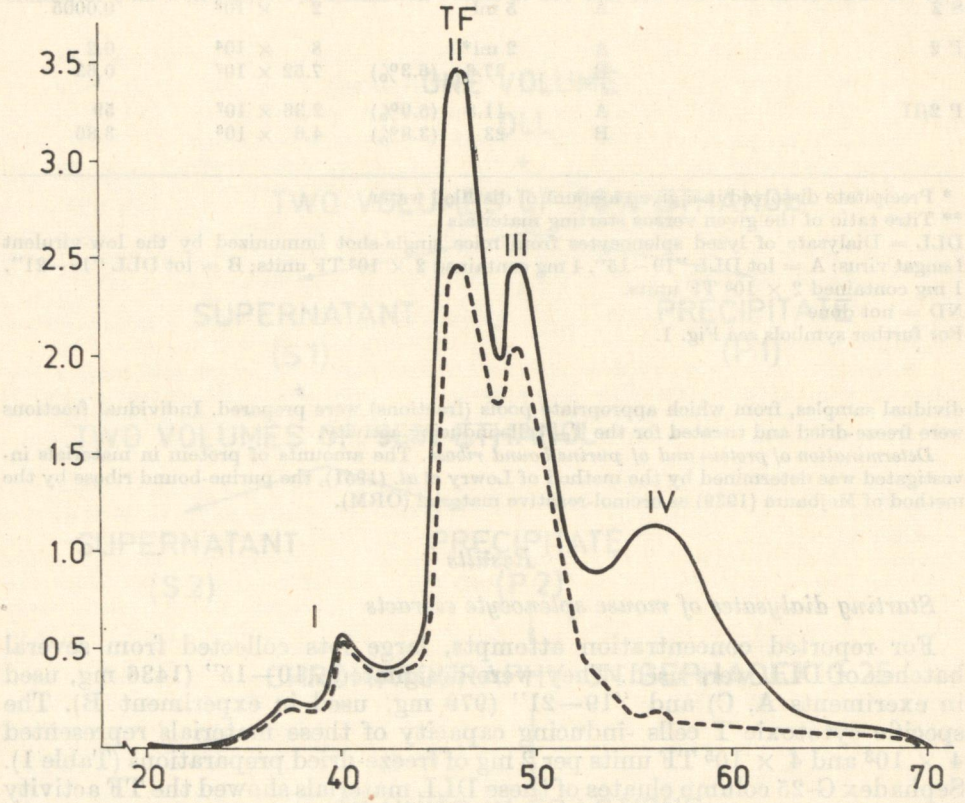

Fig. 2.

Chromatography of dialyzed spleen cell lysate (100 mg of dry weight) on a 2.5 × 90 cm Sephadex G-25 (fine) column

Eluent; bidistilled water, effluent rate 38.5 ml per hr, 2 ml samples were collected and monitored at 260 nm (————————) and 280 nm (— — — — —), respectively.

Abscissa: fraction number; ordinate: adsorbancy at given wavelengths.

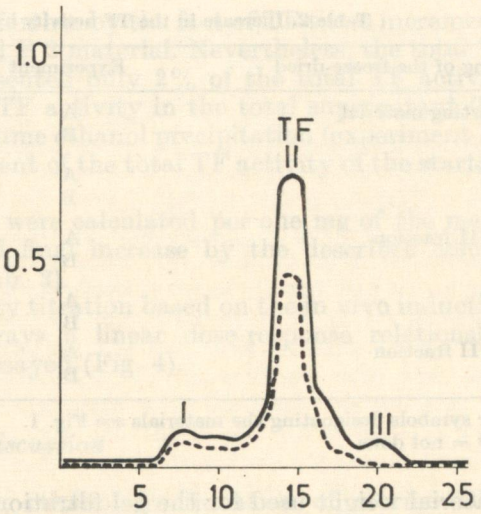

Fig. 3.
Chromatography of the precipitate obtained after addition of four volumes of ethanol to dissolved DLL
50 cm Sephadex G-25 (fine) column; eluent: bidistilled water, effluent rate 11 ml per hr, 2 ml samples were collected and monitored for adsorbance at 260 nm (———) and 280 nm (— — — —), respectively.

cent were recovered of the total TF activity, present in the starting amount of DLL in experiments A, B and C. Thus, the P 2 precipitate possessed 10—134 times higher TF activity than the P 1 precipitate.

Materials from the experiment B were analyzed for their protein and ORM contents. When compared with the crude DLL, the protein content in the P 1 and P2 materials comprised only 3.5 and 3.9 per cent of the total protein contents in the crude DLL, which was 61.8 mg in 27.75 DLL units. Interestingly, the total ORM amount in the P 1 and P 2 precipitates was not higher than 2.35 and 4.56 per cent from the total ORM amount (4.3 mg present in the starting 595 mg of DLL. The ORM content values remained to be of the same order, i.e. 7.2, 3.6 and 5.2 μg, respectively, when calculated per 1 mg of the starting and P 1 or P 2 materials.

Fractionation of P 1 and P 2 materials on Sephadex G-25 columns yielded eluates of very similar absorbance profiles, showing always three peaks (Fig. 3). The TBE-virus antigen-specific T_{cx} cells inducing activity of TF was detected exclusively in the IInd fraction of the P 1 and P 2 eluates, which contained collected samples from the well-defined peak area with the relatively high 260/280 nm absorbance ratio. As previously noted, the protein content per one mg of the freeze-dried P 2 material amounted to 20% of the protein content in one mg of the starting DLL. This fact was confirmed also by the recorded absorbance values. On the other hand, the ORM amount per one mg of material eluted in the IId fraction during the gel filtration of either the P 1 or the P 2 material (experiment B), ranged from 4.5 to 6 μg, i.e. it was very close to values, estimated in these non-chromatographed materials.

The total amount of TF units in the IInd fraction of the P 2 material surpassed that in unfractioned P 2 materials 295-times in experiment A and 6.1-times in the experiment B. In the IInd fraction 61.2 per cent of the P 2

Table 2. Increase in the TF activity by individual concentration steps

1 mg of the freeze-dried	Experiment	Titre of TF	Concentration factor
Starting material	A	2×10^3	0
	B	2×10^5	
P 1	A	ND	
	B	2×10^4	0.1
P1/II fraction	A	ND	
	B	2×10^5	0
P 2	A	ND	
	B	2×10^6	10
P2/II fraction	A	2×10^6	1000
	B	2×10^7	100

For symbols designating the materials see Fig. 1.
ND = not done

material weight used for the gel filtration was recovered, but its TF activity was absolutely higher. The total TF activity in the final products of the concentration procedure used was in individual experiments 3.86—59 times higher than the total TF activity of the whole amounts of DLL used for described investigations (Table 1). Of significance was that the total weight of the P /II fraction materials comprised only 3.8—5.9 per cent of the initial DLL amounts weights. The importance of the chromatography step in the

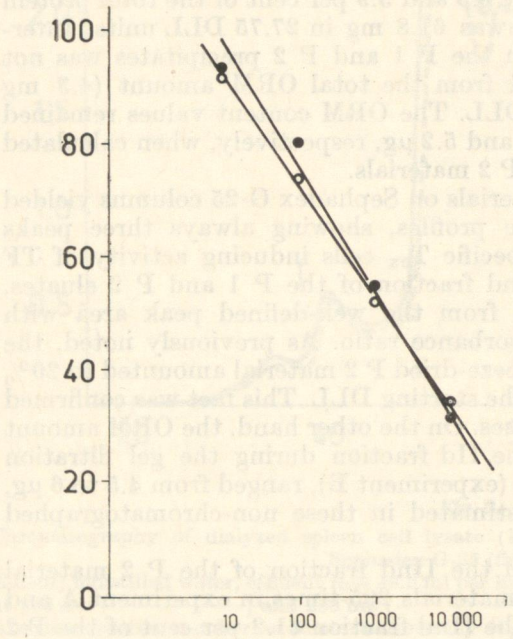

Fig. 4.
Dose-response relationship between generation of T_{cx} lymphocytes as detected by ^{51}Cr-release assay and TF activity The difference between slopes of both curves is not significant. For methodical explanation see Fig. 1.
Exp. A (○): $y = 107.82 + 7.69 \ln x$
Exp. B (●): $y = 113.37 + 8.16 \ln x$
Abscissa; dilutions of the chromatographed material; ordinate: ^{51}Cr-release (in %).

concentration process is illustrated further by the fact of TF titres increments in the IInd fraction of both P 1 and P 2 material. Nevertheless, the total TF activity in the P 1 material represented only 2% of the total TF activity in the starting DLL amount. The TF activity in the total supernatant (S2, Fig. 1), remaining after the four volume ethanol precipitation (experiment B), was negligible, being only 0.05 per cent of the total TF activity of the starting DLL amount.

When the TF activity incremets were calculated per one mg of the materials investigated, a 100—1000-fold final increase by the described combined method had been achieved (Tab. 2).

The advised method of TF activity titration based on the in vivo induction of specific T_{cx} cells, revealed always a linear dose-response relationship with chromatographed materials assayed (Fig. 4).

Discussion

As follows from data presented, ethanol fractionation of murine DLL into two precipitates, may serve as an efficient initial step for further concentration of TF activity. Actually, precipitates formed after addition of two volumes of ethanol, contained only 0.5—2% of the starting DLL total specific activity, its level in the S2 supernatant being not significant. Bulk of the activity was recovered in the precipitates formed by four volumes of ethanol. Though constituting only about 6.3% (experiment B) of the DLL dry weight, they contained 20—63.2% of its total specific activity. Nevertheless, subsequent fractionation of the precipitates by exclusion chromatography resulted in obtaining the materials with activity 3.9—59 times higher than the total activity of the original DLL, although their weight represented only 3.8—5.9% of the starting substance. These findings could reflect the differences between the kinds of molecular interactions taking place during ethanol precipitation of the TF activity-carrying substrate from the DLL solution and during gel filtration, e.g. the removal of substances hypothetically regulating or inhibiting the TF activity investigated. The gel filtration resulted in a significant increase of TF activity in either precipitate. Fractionation by ethanol — being also a partial purification — appears as unavoidable step in the reported, relatively effective procedure. This is stressed by the fact that exclusion chromatography per se of otherwise untreated DLL never yielded an increment in the TF activity, which would surpass the total activity in the specific TF material containing fraction of the eluates. Only relative concentration was achieved, total TF specific activity being 3.25 — 5.6 times lower than in the starting DLL (Mayer *et al.*, 1982). At the present state of evidence, we are inclined to assume that the ethanol precipitation and chromatography eliminate — at least partially from the murine DLL — — certain substances, interfering in a still undescribed way in the crude DLL preparation with the TF effects studied. Further investigations are necessary to elucidate what bearing the present findings may have for such a regulatory activity identification and/or disclosure of the mechanism of its action.

The frequently observed, but still unmanageable variability in the composition of individual DLL preparations is considered as an important factor influencing the final recovery of TF by the chromatography alone. However, by the described combined procedure, partially purified materials with an increased TF activity titres may be obtained from DLEs showing a low level of TF activity.

A structural model for human TF suggested by enzymatic susceptibilities (Burger *el al.*, 1979) proposes a peptide component with a phosphodiester linkage to ribonucleotide, having a free 3'hydroxyl group. The four volumes of ethanol are known to precipitate substances of mononucleotide character (Klesius, 1979). It cannot be excluded that material enriched in such a way with the TF activity substrate is more suitable for not only further concentration, but also purification by exclusion chromatography, as documented by preliminary findings of a remarkable protein content decrease in the final product with the ORM content remaining relatively constant. It is difficult to say according to the informations available, whether this ribose -bound material participates in the TF activity investigated, because no substantial difference in the amounts of ORM (within the confidence limits of the detection method used) obtained by gel filtration of the P 1 and of the P 2 materials was found (Tab. 1). However, the TF activity was lower by 2.9 $\log_{10}$ units (i.e. about 820-times) in the P1/IInd fraction. As a matter of fact, the above structural model of TF remains still a suggestion, the material subtrate of it being not known as yet.

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