Lymphocytotoxic antibodies in systemic lupus erythematosus: studies of their temperature dependence, binding characteristics, and specificity in vitro

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SUMMARY The lymphocytotoxicity of 33 lupus sera was tested against purified helper/inducer (OKT4) and cytotoxic/suppressor (OKT8) subsets of T lymphocytes at 15°C and 37°C in vitro. There was significantly less killing of both OKT4 and OKT8 cells at 37°C ($p < 0.001$ and $p < 0.01$) and the ratio of OKT4/OKT8 cell killing at 15°C (1.39 (0.73); mean (SD)) was different from that observed at 37°C (0.79 (0.42)) ($p < 0.001$). OKT4 killing was greater than OKT8 killing in 21 out of 33 sera at 15°C, while 22 of these sera showed predominantly OKT8 cytotoxicity at 37°C. The relation between the OKT4/OKT8 cell ratio and OKT4/OKT8 serum killing was examined in 22 patients at both temperatures: a significant inverse correlation was observed at 37°C ($r = -0.53$; $p = 0.015$) but not at 15°C ($p > 0.05$). The addition of metabolic and cytoskeletal inhibitors increased cytotoxicity at 37°C, but not IgM surface binding. A Scatchard binding analysis of the reaction at 15°C showed that large numbers of antibody molecules were bound to both subsets, with a low average dissociation constant of $\leq 6 \times 10^{-8}$ mol/l, and electrophoretic blotting indicated that the target surface antigens varied in type and number among individual lymphocytotoxic sera. The demonstration of temperature dependent, tight binding between lymphocytotoxic antibody and variable antigens on the T cell surface emphasises the potential for this phenomenon to affect lymphocyte function in vivo in patients with systemic lupus erythematosus.

Key words: lymphocyte surface antigens, Scatchard analysis, metabolic inhibition.

More than 90% of sera from patients with systemic lupus erythematosus (SLE) show toxicity for normal peripheral blood lymphocytes in vitro.¹ This lymphocytotoxic activity (LCA) resides predominantly (but not exclusively) in the IgM fraction and is maximal at 15°C. The temperature dependence is thought to represent a compromise between the warm incubation requirements of complement mediated lympholysis and the optimal binding conditions for IgM class lymphocyte antibody (i.e., 4°C).² Studies of the specificity of LCA in SLE have produced conflicting reports. Some workers have shown reactivity against the suppressor T cell subset

(as defined by either functional testing or surface markers),³ while others have observed cytotoxicity against the helper/inducer population.⁴ A recent study showed that the circulating T cell subset ratio correlated with the subset specificity of LCA in lupus sera⁵; observations were made only at 15°C, however. Little is known about the specificity of serum LCA at 37°C, or indeed the influence of assay temperature on subset killing. The kinetics of antibody binding and the nature of the surface antigens involved are also unclear. These issues must be resolved in order to ascribe more confidently a physiological role to this reaction.

In this study we have examined in detail the temperature dependence and specificity of the reaction. Scatchard analysis was used to estimate the dissociation constant of the interaction between

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lymphocyte and autoantibody, as well as the quantity of antibody bound. The ability of various metabolic inhibitors to restore killing at 37°C was also investigated. Finally, we used electrophoretic blotting techniques to demonstrate the variability, within a group of serum samples with high titre LCA, of the number and molecular weight of lymphocyte surface antigens reactive with these IgM autoantibodies.

Patients and methods

PATIENTS

Serum samples were obtained from 33 patients with SLE⁶ and eight healthy volunteers. Aliquots were stored at -20°C until analysed.

MEASUREMENT OF LCA

T lymphocytes were purified from peripheral blood by Ficoll-Hypaque centrifugation after fractionation over nylon wool. Subsets were prepared by incubation of purified T cells with monoclonal anti-OKT8 or anti-OKT4 (Ortho) antibodies (5 µl/10⁶ cells in complement fixation diluent (CFD; Oxoid)) for 30 minutes at 20°C, followed by the addition of an equal volume of rabbit serum (as a source of complement) and incubation for 90 minutes at 37°C. Lysed cells were removed by Ficoll-Hypaque centrifugation. Cell purity was approximately 90% as confirmed by immunoperoxidase staining with anti-OKT4 and anti-OKT8 antibodies and peroxidase conjugated antimouse immunoglobulin (Dako).

Serum samples were tested for LCA against T cells and purified subsets at both 15°C and 37°C. The procedure was a modification of that described by Terasaki and McClelland.⁷ Specifically, 20 µl of cell suspension (1×10⁶ cells/ml) in CFD was premixed with 20 µl of patient or control serum before the addition of 40 µl of rabbit serum. The mixture was incubated for 60 minutes at 37°C or 180 minutes at 15°C before the determination of percentage cell lysis by a standard trypan blue exclusion technique.

ANALYSIS OF IgM BINDING TO THE LYMPHOCYTE SURFACE

Affinity purified sheep antihuman IgM (Silenus) was labelled with ¹²⁵I (Amersham) by the lactoperoxidase method.⁸ This labelled antibody showed only one precipitin line against normal human serum (NHS) in a standard double diffusion assay in agarose.

Purified lymphocytes and test sera were diluted in 10% de complemented fetal calf serum (Commonwealth Serum Laboratory) in CFD with 0.05% sodium azide. Duplicate tubes containing 200 µl of lymphocytes (5×10⁶/ml of OKT3, OKT4, or OKT8

positive cells) were incubated with an equal volume of diluted serum at 15°C for two hours. (Preliminary experiments showed that binding equilibrium was reached under these conditions.) The cells were then layered onto 2 ml of 5% bovine serum albumin in CFD/azide, and unbound molecules separated by centrifugation at 1000 g for five minutes. The cell pellet was resuspended in 400 µl of a 1 in 100 dilution of ¹²⁵I anti-IgM in fetal calf serum/CFD/azide and incubated for two hours. Finally, the cells were again layered into 5% bovine serum albumin, separated by centrifugation, and the tip of the polystyrene tube containing the cellular pellet excised and counted in a gammacounter. Molecules of IgM bound to the cell surface were determined by comparison with the binding of labelled antibody to known amounts of IgM. (Complexed antibody was separated from unbound molecules by precipitation with 10% polyethylene glycol (mol. wt 6000) and centrifugation at 2000 g for 30 minutes.

Control experiments showed negligible binding in cell free polystyrene tubes and all solutions were centrifuged at 2200 g for 30 minutes before assay to remove aggregated material. The reversibility of IgM surface binding was determined by the prolonged (i.e., up to eight hours) incubation of cells in human serum free medium after preincubation with serum with positive LCA (as above).

IgM concentration was measured by radial immunodiffusion using a standard control serum (Calbiochem). Scatchard analysis was used to calculate the dissociation constant (K_d), while the concentration of binding sites per cell was determined with the additional use of Avogadro's number and the quantity of cells per tube.

CHARACTERISATION OF LYMPHOCYTE SURFACE ANTIGENS

Lymphocytes were washed in CFD/azide and incubated with normal or SLE sera (10⁸ cells/40 µl serum) for 60 minutes at 4°C. The cells were resuspended in an additional 200 µl CFD/azide, again separated by centrifugation, and the lymphocytotoxic reactivity of the supernatant (i.e., of normal and SLE sera) compared with that of unabsorbed normal and patient sera. These absorbed samples were also used as probes for the experiments described below.

Samples of normal donor lymphocytes (1.5×10⁶) were boiled in 5 ml of reducing sodium dodecyl sulphate (SDS) buffer, passed through a 0.22 µm filter (Amicon) and centrifuged for 60 minutes at 35 000 rpm before polyacrylamide gel electrophoresis⁹ in SDS on 12% agarose gels (16×0.1 cm). One track was reserved for SDS-6H (Sigma) molecular weight markers. Samples were electro-

phoresed overnight at a constant 60 volts. Electrophoretic transfer to nitrocellulose was performed according to the method of Towbin *et al*¹⁰ in 0.025 M trometamol (TRIS)-glycine buffer with 20% v/v methanol for four hours at 70 V and adjacent strips of nitrocellulose then probed with absorbed or non-absorbed sera. Specifically, aliquots of these sera (20 µl/4 ml of incubation buffer) were incubated and shaken overnight before the detection of bound IgM with monospecific rabbit antibody (Dako) and an affinity purified goat antirabbit alkaline phosphatase conjugate (Bio Rad). Both antibodies were used at room temperature for 60 minutes in incubation buffer (i.e., 0.02 M trometamol buffer with 0.5 M NaCl, 1% w/v gelatine, and 0.05% Tween 20). Colour development was performed with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in a bicarbonate buffer at pH 9.8. Experiments with strips stained with decreasing amounts of serum showed that colour intensity was responsive to the serum concentration for most bands at this proportion of serum to lymphocyte antigen.

EFFECT OF METABOLIC INHIBITORS ON LYMPHOCYTE KILLING IN VITRO

The metabolic inhibitors azide, fluoride, and arsenite were prepared as the sodium salts in CFD and tested for intrinsic lymphocytotoxicity before use in experiments. The highest concentrations that gave minimal toxicity (i.e., <5%) were used—0.05% NaF, 0.05% NaN₃, and 0.01% NaAsO₂. Purified T cells were washed three times in CFD or CFD/inhibitor and resuspended in the same solution at a concentration of 2×10^6 cells/ml. They were then incubated with either normal or SLE sera at 15°C (180 minutes) or 37°C (60 minutes) in the presence of rabbit serum (decomplemented for some experiments), and the percentage cell lysis determined by standard methods. Colchicine, lidocaine, and dibucaine were also prepared in CFD and incubation conditions selected by the same criteria as those used above: 2×10^6 T cells/ml were incubated with 5×10^{-5} M colchicine, 50 µg/ml lidocaine, and 5 µg/ml dibucaine.

The effect of metabolic inhibitors on the binding of IgM to the lymphocyte surface was measured by Scatchard analysis.

STATISTICAL ANALYSIS

A paired Student's *t* test was used to calculate the significance of differences in the binding kinetics. Scatchard plots were analysed by linear regression and calculation of the 'r' coefficient.

The difference between percentage killing of OKT4 and OKT8 cells at 15°C and 37°C was analysed by a Wilcoxon match pair test. The difference between OKT4/OKT8 killing at 15°C and

37°C was analysed by an unpaired Student's *t* test, and the correlation between OKT4/OKT8 cell ratio and OKT4/OKT8 killing at 15°C and 37°C was also examined by linear regression analysis with calculation of the r coefficient.

Results

TEMPERATURE DEPENDENCE OF CYTOTOXICITY

The percentage lymphocytotoxicity of 33 SLE sera, tested against purified OKT4 positive cells, was significantly greater at 15°C than at 37°C: 53 (26)% (mean (SD)) and 18 (10)% respectively ($p < 0.001$). In comparison, mean OKT8 cell killing was 45 (28)% and 28 (18)% ($p < 0.01$). The ratios of OKT4/OKT8 cells killed by test sera at 15°C and 37°C were significantly different: 1.39 (0.73) at 15°C (mean (SD)) and 0.79 (0.42) at 37°C ($p < 0.001$). At 15°C, 64% (21/33) of sera showed OKT4 killing greater than OKT8 killing, while at 37°C, 67% (22/33) of sera showed predominantly OKT8 cytotoxicity. There was also a significant inverse correlation between the OKT4/OKT8 serum killing and the patients' OKT4/OKT8 cell ratio at 37°C ($r = -0.53$, $p = 0.015$), but not at 15°C.

ANALYSIS OF IgM BINDING TO TARGET LYMPHOCYTES

Preliminary experiments showed (a) The precipitin reaction between IgM (contained in dilutions of NHS) and ¹²⁵I anti-IgM was linear for the range 0–0.15 µg ($r = 0.98$). (b) The binding between IgM coated T lymphocytes (i.e., by prior incubation with sera with positive LCA) and radiolabelled anti-IgM was maximal after two hours' incubation and with a 1/100 dilution of antibody. IgM from cytotoxic sera, after chromatographic separation from IgG, gave similar results. (c) There was a linear relation between surface bound IgM and the proportion of target lymphocytes that had been pre reacted with lymphocytotoxic lupus sera (Fig. 1).

Effect of incubation temperature on binding of lymphocytotoxic antibody

Fig. 2 shows data for a typical lupus serum. Binding was maximal for all dilutions (i.e., 1/10, 1/100, 1/1000) after two hours' incubation at either 4°C or 15°C. There was variability in binding at 37°C, and all sera showed a substantial decrease in bound IgM compared with that observed at lower temperatures. NHS showed a similar pattern of temperature dependence for antibody binding, although at each concentration less antibody was bound from NHS than from SLE sera.

Fig. 1 Relation between binding of IgM to the lymphocyte surface and the proportion of target cells that had been prereacted with lymphocytotoxic lupus sera. Surface IgM is expressed as a percentage of that observed when all target cells had been prereacted with cytotoxic serum. Mean (SD) values are shown.

Fig. 2 Effect of variations in incubation time (h) and temperature (°C) on the amount of IgM bound to the lymphocyte surface. Results for one typical lupus serum are shown (expressed in terms of maximum IgM binding attained). ●=1/10 dilution, ◆=1/100 dilution, ▲=1/1000 dilution.

Dissociation of surface bound IgM

Fig. 3 shows the effect of prolonged incubation (at 15°C) of IgM coated T lymphocytes in human serum free medium. Cells that had been preincubated with either NHS or lupus sera showed an initial rapid fall in bound antibody (i.e., over the first two hours) followed by a slower fall over the next six hours. Overnight incubation resulted in unacceptably high cell mortality.

Measurement of dissociation constant (Kd) and surface IgM concentration

Fig. 4 shows the effect of variation of serum concentration (i.e., NHS and SLE) on IgM binding to OKT4 and OKT8 positive cells (at 15°C); an initial increase in IgM binding was followed by a plateau. Table 1 shows values obtained for Kd and surface IgM concentrations with six lupus sera and two NHS pools. The dissociation constants for both OKT4 and OKT8 positive cells were similar for the two groups of sera, though the concentration of surface bound IgM was generally much greater for cells incubated with SLE sera (Table 1).

DETECTION OF IgM REACTIVE LYMPHOCYTE SURFACE ANTIGENS BY ELECTROBLOTTING

When different pools of normal lymphocytes were probed with several SLE and normal sera consistent patterns for each serum were found, but between different sera there were differing patterns of IgM reactive lymphocyte antigens. While some bands

Fig. 3 Effect on surface bound IgM of prolonged incubation of prereacted T lymphocytes in serum free medium. Mean (SD) values are shown for cells preincubated with SLE sera (●) and normal human serum (○).

were shared between SLE and normal sera, additional antigens (generally in higher concentration) were observed with the SLE sera. Pre-absorption of six SLE and six normal sera with normal lymphocytes led to a significant reduction in some band densities (on nitrocellulose) in SLE sera but not in normal sera (Fig. 5). Three of the six SLE serum samples showed a loss of one band of 90 000 kilodaltons and another three showed a loss of a band of approximately 34 000 kilodaltons. No single band was lost by all of the six sera after preabsorption (Table 2).

Fig. 4 Effect of variation of IgM concentration on the amount of IgM bound to the lymphocyte surface. ●, ○=OKT4, OKT8 cells incubated with SLE sera; ◆, ◇=OKT4, OKT8 cells incubated with normal human serum.

Table 1 Binding characteristics of IgM lymphocytotoxic antibodies for OKT4 and OKT8 positive cells

Serum	OKT4 cells			OKT8 cells		
	Kd (mol/l)	IgM molecules/OKT4 cell*	LCA on OKT4 cells†	Kd (mol/l)	IgM molecules/OKT8 cell*	LCA on OKT8 cells†
NHS 1	2.5×10 ⁻⁸	12 700	0	3.2×10 ⁻⁸	7 100	0
2	2.9×10 ⁻⁸	7 300	0	1.9×10 ⁻⁸	19 300	3
SLE 1	2.7×10 ⁻⁸	11 200	29	2.1×10 ⁻⁸	178 600	25
2	6.0×10 ⁻⁸	46 100	66	3.5×10 ⁻⁸	74 500	24
3	3.2×10 ⁻⁸	96 100	28	5.3×10 ⁻⁸	96 900	21
4	1.6×10 ⁻⁸	50 300	62	5.8×10 ⁻⁸	49 700	24
5	2.3×10 ⁻⁸	60 600	18	5.8×10 ⁻⁸	31 100	9
6	1.7×10 ⁻⁸	29 700	22	3.7×10 ⁻⁸	31 000	18

*Assuming equal numbers of molecules per cell.
†Expressed as percentage cell lysis. Normal≤5%.

EFFECT OF METABOLIC INHIBITORS ON LYMPHOCYTOTOXIC ACTIVITY

Table 3 shows the LCA of six normal sera and six SLE sera when incubated with normal T lymphocytes in the presence of azide, fluoride, arsenite, colchicine, lidocaine, and dibucaine. There was a significant increase in the percentage of cells lysed at 37°C by

Table 2 Molecular weights of surface antigens reactive with IgM from SLE sera

Serum					
A	B	C	D	E	F
40 000	125 000	110 000	90 000	90 000	90 000
	62 000	65 000	35 000		34 000
	19 000	33 000	16 500		27 000
		11 500	13 500		
		10 000			

Table 3 Effect of various inhibitors on lymphocytotoxicity at 15°C and 37°C†

	Normal sera		SLE sera	
	15°C	37°C	15°C	37°C
CFD‡	8	8	76	15
Azide	8	6	70	25*
Fluoride	7	7	67	29***
Arsenite	6	8	70	37***
Colchicine	7	7	84	24**
Lidocaine	12	6	78	27**
Dibucaine	10	8	77	31***

*p<0.025; **p<0.002; ***p<0.001 (compared with complement fixation diluent).

†n=6 for each group. Data shown as mean percentage of cells lysed.

‡CFD=complement fixation diluent.

SLE sera when incubated in the presence of each of these inhibitors compared with cells suspended in CFD alone. The cytotoxicity of the inhibitors, with and without test sera and in the presence of de complemented rabbit serum, was not significant. In general, these agents did not significantly affect

the amount of IgM bound to the lymphocyte surface, though the amount bound at 37°C was significantly less than at 15°C ($p < 0.05$ for all incubation media, with both NHS and SLE sera). There was no correlation between the amount of IgM binding to the cell surface after incubation with SLE or normal serum and the lymphocytotoxicity of that serum.

Discussion

This study demonstrated significant differences in the T lymphocyte subset specificity of lymphocytotoxic lupus sera at 15°C and 37°C. At 15°C (the temperature at which most studies are performed) toxicity was greater for OKT4 cells. No significant correlation was observed between the patients' OKT4/OKT8 cell ratio and OKT4/OKT8 killing, though this has been reported.⁵ In contrast, cytotoxicity at 37°C was greater for OKT8 cells, and this was associated with a significant reduction in the fraction of OKT4 and OKT8 cells killed. There was also a significant inverse correlation between OKT4/OKT8 serum killing and the OKT4/OKT8 cell ratio (i.e., at 37°C). The data showed that the lower cytotoxicity towards unfractionated T cells at 37°C (as compared with 15°C) was due predominantly to a reduction in toxicity for OKT4 positive cells. This finding of significant OKT8 cytotoxicity at 37°C supported the view that LCA may contribute to the suppressor cell deficiencies observed in autoimmune diseases. Some groups have shown that antagonism of suppressor cell function resides in the IgG fraction of SLE serum, which includes the warm reactive lymphotoxins.¹¹ Kinetic studies were performed at 15°C to allow direct comparison with the standard lymphocytotoxic assay. At 37°C, inhibition of shedding or internalisation of lymphocytotoxin, or both, regardless of the presence of inhibitors of metabolism and cytoskeletal function, appeared incomplete as both IgM binding and lymphocyte killing were significantly lower than observed with the same sera at 15°C. These inhibitors were able to increase killing at 37°C compared with that observed with CFD alone, however. A cumulative, non-specific toxic effect of these reagents is unlikely as they did not change the toxicity of normal sera at 15°C and 37°C and of SLE sera at 15°C. Winfield *et al* used semiquantitative data from a fluorescence activated cell sorter in similar experiments, and they also found no recovery of IgM binding at 37°C in the presence of cytoskeletal and metabolic inhibitors. They did not investigate the influence of these reagents on cell killing, however.

Before the Scatchard analysis the interaction between IgM antibody and lymphocyte surface was

Fig. 5 IgM reactive lymphocyte antigens in normal (track 1) and SLE (track 2—patient B, Table 2) sera, as separated on reduced sodium dodecyl sulphate gels. The left track was derived from unabsorbed serum, the right from serum absorbed with viable cells. Bands (→) at 125, 62, and 19 kilodaltons are much reduced in B absorbed serum. Molecular weight markers are shown as kilodaltons (Kd).

shown to be reversible (Fig. 3), with comparable rates of dissociation being found in both normal and lupus derived IgM.

A quantitative analysis of IgM binding to the surface of subsets of normal T lymphocytes showed that both normal and SLE sera contained molecules that bound with high affinity (i.e., Kd of $1-6 \times 10^{-8}$ mol/l). SLE sera contained a much higher proportion of these molecules, however. IgM in lupus sera showed a saturable binding response, and at the highest concentration of free IgM there was actually a decrease in surface bound antibody. This was consistent with observations in infectious mononucleosis,¹³ which showed that dilution of lymphocytotoxic sera could lead to enhanced (rather than reduced) lymphocyte killing. This was postulated to result from non-specific inhibition of binding of cytotoxic molecules at high serum concentrations. The binding data for NHS and SLE sera gave values for Kd of $1-6 \times 10^{-8}$ mol/l and up to 178 000 IgM molecules bound per cell. No significant difference between binding of IgM to OKT4 and OKT8 positive cells was apparent. Bound/free ratios were <0.01 , suggesting that only a small proportion of IgM was able to bind tightly to the lymphocyte surface, possibly leading to overestimation of the Kd. It should also be emphasised that the calculation of the data in Table 1 assumed an equal number of sites per cell, though individual cell surfaces probably have different capacities (see below). As we found half saturation of binding to occur at $<10^{-7}$ mol/l in vitro, most binding sites on lymphocytes would be expected to be occupied at the high concentration of IgM found in serum, and, indeed, Winchester *et al* noted that a high percentage of lymphocytes in some patients were coated with immunoglobulin.² A selective decrease in brightly stained T cells as measured by fluorescence activated cell sorter has been described,¹⁴ emphasising the potential for in vivo processes to modify the interaction between T cell and lymphocytotoxin.

A small number of the IgM reactive lymphocyte molecules, detected by immunoblotting techniques, were identified as surface antigens, i.e., they were absorbed by living cells under conditions which remove virtually all lymphocytotoxic activity from test serum. Solid phase immunoblotting methods have been criticised on the grounds that they may detect antibody/antigen interactions of low affinity, and thus of questionable importance in vivo.¹⁵ This is because of the amplification of assay sensitivity by the immobilisation of antigen, which leads to an increase in avidity. Some of the interactions detected probably are of low affinity, though autoantibodies are commonly found in normal serum.¹⁶ We were justified in attributing biological importance to the

interactions reported in Table 2, however, because (a) IgM binding to the lymphocyte surface had an average Kd of $\leq 6 \times 10^{-8}$ mol/l, and (b) the removal of IgM antibody by interaction with living cells was, by definition, a physiological process. In some cases a significant reduction rather than a total removal of a band was seen. This was attributable to partial shedding of the multivalent antibody-antigen complex, which was not preventable even at 4°C in the presence of azide. Other low affinity interactions may also have been present. Each SLE serum sample used in this study of surface antigens had lymphocytotoxicity of $>80\%$, yet showed wide differences in the numbers and molecular weights of surface reactive autoantibodies. This explains the lack of a close correlation between LCA and IgM binding which we have demonstrated in this report.

Further work will clarify the importance of the various surface antigens through their association with disease severity or degree of lymphocyte dysfunction (as has been done for the many intracellular autoantigens found in SLE¹⁷). The differences noted between various SLE sera emphasised the view that lymphocyte autoantibodies do not, in this disease, result from non-specific polyclonal activation of the B cell repertoire.

The demonstration of tight binding between LCA and each lymphocyte subset and the observed correlation between LCA specificity and OKT4/OKT8 cell ratio provide further evidence for the potential importance of this reaction in vivo. The data also demonstrate the influence of assay methodology on the analysis of LCA behaviour in vitro. Metabolic inhibition caused an increase in cell killing at 37°C without a significant change in IgM binding; this presumably reflects more efficient complement function at physiological temperatures. Finally, the observation that different surface antigens are targeted in individual patients suggests the potential for a range of functional effects on the T lymphocyte and a means of monitoring clinical lupus activity.

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