Vibrio cholerae O1 Ogawa detoxified lipopolysaccharide structures as inducers of cytokines and oxidative species in macrophages

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Multidrug resistance in several strains of *Vibrio cholerae* has encouraged anti-cholera vaccine developmental attempts using various subcellular moieties. In order to examine the immunological efficacy of detoxified LPS (dLPS)-derived saccharide immunogens, *ex vivo* activation of mouse peritoneal macrophages (MΦs) was investigated. The immunomodulatory effect was evaluated via induction of the pro-inflammatory cytokines tumour necrosis factor- α , interleukin (IL)-1 α and IL-6 and acceleration of nitric oxide (NO) and reactive oxygen species (ROS). Immunologically active structures triggered mouse peritoneal MΦs to secrete cytokines and release NO/ROS, even at concentrations as low as 12.5 $\mu\text{g ml}^{-1}$. It was found that the O-specific polysaccharide moiety was more immunologically efficient than the glycolipid one, probably due to the position of 3-deoxy-D-manno-octulosonic acid. The results revealed effective structure–immunomodulating relationships of dLPS-derived moieties that are desirable in subcellular anti-cholera vaccine design.

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INTRODUCTION

Intestinal cholera infection is normally regarded as a non-invasive disease, because the causative agent, *Vibrio cholerae*, primarily affects the intestinal lumen. The existence of strains of *V. cholerae* resistant to several antibiotics and the increase in multidrug resistance have encouraged anti-cholera vaccine developmental attempts (Yamamoto *et al.*, 2000; Roychowdhury *et al.*, 2008). LPS, the major constituent of the outer membrane of Gram-negative bacteria, consists of a hydrophobic toxic lipid A, an outer and inner core oligosaccharide (core antigen or R-polysaccharide) and a somatic antigen [O-specific polysaccharide (O-SP)]. These LPS domains represent the main areas of interest in cholera vaccine design (Chatterjee & Chaudhuri, 2003, 2006; Erridge *et al.*, 2002). A number of subcellular anti-cholera conjugate vaccines are currently being researched and/or are in pre-clinical development (Boutonnier *et al.*, 2001; Chernyak *et al.*, 2002; Gupta *et al.*, 1992; Kossaczka *et al.*, 2000).

Cholera infections frequently induce specific mucosal pathogen-associated immune responses. Monocyte-derived

MΦs represent the first line of host defence and are one of the most important cells acting in innate immune responses. Several groups of MΦ surface molecules are involved in specific interactions with LPS, particularly in the processes of cell recognition and activation (Chaby, 2004; Janeway & Medzhitov, 1999; Alexander & Rietschel, 2001), and induced secretion of pro-inflammatory cytokines has been documented (Scheibenbogen & Andreessen, 1991; Haefner-Cavaillon *et al.*, 1988; Rabehi *et al.*, 2001; Cavaillon, 1994). LPS-induced cell activation results in the generation of nitric oxide (NO) and reactive oxygen species (ROS). Both ROS and NO participate in host defence biological activities. It has been proposed that redox signalling plays an important role in the regulation of inflammatory responses (Forman & Torres, 2002). Severe immunological inflammation or Gram-negative septic shock with exaggerated cytokine production could be manifested as a consequence of intact LPS overactivation of innate immunity. Pro-inflammatory cytokine overproduction is undesirable in cholera vaccine design. As lipid A exerts endotoxic activities, detoxification of LPS by lipid A elimination represents the first step in the preparation of a subcellular anti-cholera vaccine.

The aim of our study was to evaluate structure–immunomodulating relationships of novel *V. cholerae* detoxified LPS (dLPS)-derived moieties and the influence of different detoxification approaches on their effectiveness as immunomodulators.

Abbreviations: DeOAc-LPS, de-O-acetylated glycolipid; dLPS, detoxified LPS; EU, endotoxin unit; GM-CSF, granulocyte–macrophage colony stimulating factor; IFN, interferon; IL, interleukin; KDO, 3-deoxy-D-manno-octulosonic acid; MΦ, macrophage; O-SP, O-specific polysaccharide; ROS, reactive oxygen species; TNF- α , tumour necrosis factor- α .

METHODS

Preparation of LPS. A dried acetone precipitate of *V. cholerae* O1 Ogawa El Tor was obtained from Dr Milan Seman (Vibrio Collection, Comenius University, Bratislava, Slovakia). LPS was extracted from the cells according to the method of Westphal & Jann (1965).

Detoxification of LPS and preparation of the glycolipid fraction. For partial delipidation, LPS (100 mg) was dried over phosphate pentoxide and suspended in 10 ml anhydrous hydrazine (Sigma-Aldrich Fine Chemicals) (Kondo *et al.*, 1991). The solution was placed in a 37 °C water bath for 2 h. Cold acetone was added slowly to 90 % to precipitate the polysaccharide. Residual LPS was removed by ultracentrifugation. The final product was passed through a G-50 Sephadex column (2.5 × 50 cm; Pharmacia) in pyrogen-free water, sterile-filtered and freeze-dried. The de-O-acetylated glycolipid product, designated DeOAc-LPS, contained less than 10 endotoxin units (EU) μg^{-1} and <1 % protein or nucleic acid.

Preparation of core-attached O-specific polysaccharide. The LPS was suspended in 5 % acetic acid and heated at 100 °C for 2 h. After cooling and pH neutralization, the mixture was ultracentrifuged at 64 000 g for 5 h to remove lipid A. The supernatant was dialysed against water, passed through a Sephacryl (S-200) column, sterile-filtered (0.2 μm) and freeze-dried. The product was designated O-SP.

Limulus amoebocyte lysate test. The *Limulus* amoebocyte lysate assay for endotoxin estimation was performed according to the technical information bulletin for the E-TOXATE kit (Sigma).

Animals. Male ICR mice aged 8–12 weeks were obtained from the breeding facility of the Institute of Experimental Pharmacology, Slovak Academy of Sciences (Dobrá Voda, Slovakia). Animal experiments were conducted in compliance with Good Laboratory Practice/Organisation for Economic Co-operation and Development guidelines, according to the ethical guidelines issued by the Institute of Virology, Slovak Academy of Sciences.

Preparation, culture and induction of MΦs. To stimulate peritoneal MΦ proliferation, mice were injected intraperitoneally with 2 ml sterile 5 % thioglycolate broth (Difco Laboratories) (Park & Rikihisha, 1991). Five days later, the mice were sacrificed by cervical dislocation, and MΦs were removed by peritoneal lavage using Hanks' balanced salt solution. The cells were washed by centrifugation, and 5×10^6 cells were resuspended in RPMI 1640 with L-glutamine supplemented with heat-inactivated fetal bovine serum and placed in each well of a 24-well tissue culture microplate (Sarstedt). Plates were incubated for 2 h at 37 °C in a humidified atmosphere of 5 % CO_2 . After the removal of non-adherent cells, at least 96 % of the adherent cells, which were used in further experiments, were MΦs according to morphological and phagocytic criteria (Akagawa & Tokumaga, 1985). The medium was replaced with 0.5 ml fresh medium containing LPS-derived stimulants. After cultivation, the supernatants were collected and stored at –70 °C.

Immunophenotyping of peritoneal lavage MΦs. For immunocytometric assays, cells were stained directly from peritoneal lavage (single or dual staining) and evaluated by flow cytometry using a FC 500 flow cytometer (Beckman Coulter) equipped with a 488 nm argon laser and a collinear 637 nm helium/neon laser, running under CXP software. For each sample, a histogram of 10^4 fluorescent cells was generated and analysed. The following rat anti-mouse monoclonal antibodies were applied: fluorescein isothiocyanate-conjugated CD11b (Antigenix America) and phycoerythrin-conjugated CD14 (BD Pharmingen). The appropriate antibody isotype-negative controls (anti-IgG1 or anti-IgG2a) were used separately. The conjugated monoclonal antibodies (5 μl) and peritoneal lavage samples (50 μl)

were added to 5 ml sterile Falcon tubes (Becton Dickinson) and incubated for 30 min in the dark at 4 °C. The lysis of erythrocytes was performed with 250 μl OptiLyse C lysing solution (Immunotech). Lysis was stopped after 30 min of incubation in the dark at 4 °C by the addition of PBS (pH 7.2) and the samples were evaluated by dual-colour flow cytometry.

Preparation of the polysaccharides. Solutions of the polysaccharide compounds O-SP and DeOAc-LPS were prepared at concentrations of 12.5, 25 and 50 $\mu\text{g ml}^{-1}$ in complete RPMI 1640 and sterilized by filtration through a 0.22 μm membrane (Millipore).

Determination of cytokines. The induction of tumour necrosis factor (TNF)- α , interleukin (IL)-1 α and IL-6 was measured in MΦ cell culture supernatants after 3, 6 and 24 h of cultivation with O-SP or DeOAc-LPS, using a flow cytometry fluorescent bead-based multiplex assay [Mouse Th1/Th2 10plex FlowCytomix Multiplex for quantitative detection of granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)- γ , IL-1 α , IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF- α ; Bender MedSystems]. Cytokine production of unstimulated cell populations was used to determine the baseline values.

Quantitative determination of ROS. MΦ cell culture supernatants that had been cultivated for 3, 6 and 24 h with O-SP or DeOAc-LPS were assayed for their total content of free radicals. A reagent kit based on the ability of chlorophyllin to transfer electrons due to its electron-rich double-bonds structure (Free radicals kit; Sevapharma) was used. The quantification of free radicals was determined using a calibration based on an $\text{Fe}^{2+}/\text{Fe}^{3+}$ reaction and was expressed as $\text{mmol Fe}^{2+} \text{ l}^{-1}$. ROS production of unstimulated cell populations was used to determine the baseline value.

Quantitative determination of NO_2^- production by MΦs. This assay was carried out as described by Ding *et al.* (1988). Briefly, MΦ cell culture supernatants collected after 3, 6 and 24 h of incubation with O-SP or DeOAc-LPS at different concentrations were used in the assay. A microtitre plate was used with standard NaNO_2 solutions (serial dilutions from 0.25 mM) and equal volumes of the tested supernatants. Freshly prepared Griess reagent was added to each well and the A_{550} was measured. NO_2^- production of unstimulated cell populations was used to determine the baseline value.

Statistics. The results are presented as means $\pm$ SEM. All data were statistically analysed by a one-way analysis of variance and a Bonferroni test to determine differences within the groups. *P* values less than 0.05 were considered to be significant. Analyses were performed using OriginPro 7.5 software (OriginLab).

RESULTS AND DISCUSSION

Detoxification of LPS represents the first step in the creation of safe, lipid A-free, LPS-derived immunogenic derivatives. In our experiments, two detoxification procedures were applied, acid and base hydrolysis. Two potent immunogens, O-specific polysaccharide attached to the core polysaccharide (O-SP) and glycolipid (DeOAc-LPS), were prepared. The latent toxicity of both constructs was determined using a *Limulus* amoebocyte lysate test. Both preparations were found to have less than 10 EU μg^{-1} . The induction of pro-inflammatory cytokine secretion and NO and ROS generation was studied using mouse peritoneal MΦs *ex vivo*. We observed a pattern of TNF- α , IL-1 α and IL-6 dose-dependent stimulated release in murine intra-

peritoneal MΦs from mice pre-stimulated with thioglycolate broth. As a consequence of pre-stimulation, a 2.05-fold increase in the peritoneal lavage monocyte/MΦ population in comparison with unstimulated animals ($89.7\% \pm 0.94$ CD11b⁺ CD14⁺ cells vs $43.7 \pm 0.63\%$) was detected by two-colour flow cytometry analysis. The actual morphology and cell counts were determined microscopically after staining with Türk's solution. The MΦ cell monolayer was incubated in medium containing dLPS-derived immunogens. Spontaneous cytokine release from unstimulated cells was used as a negative control. The activation of adherent cells with the two immunogens resulted in the secretion of the pro-inflammatory cytokines TNF- α , IL-1 α and IL-6. The other cytokines in the Th1/Th2 multiplex panel (GM-CSF, IFN- γ , IL-2, IL-4, IL-5, IL-10 and IL-17) were negative.

To evaluate the effect of the dLPS-derived immunogens on pro-inflammatory cytokine secretion, the method was carried out using different concentrations of the immunogens (12.5 – $50\text{ }\mu\text{g ml}^{-1}$) (Figs 1 and 2). A concentration of $12.5\text{ }\mu\text{g ml}^{-1}$ was found to be sufficient for both substances to produce a significant induction of cytokine secretion ($P < 0.001$).

Increasing concentrations of the polysaccharide and glycolipid moieties resulted in a dose-dependent increase

in induced secretion of the pro-inflammatory cytokines IL-6 and TNF- α . In contrast, IL-1 α secretion appeared to be dose-independent, with the maximum stimulation being reached at $25\text{ }\mu\text{g ml}^{-1}$. In this case, both immunogens exhibited a similar dose response. The extent of cytokine secretion varied with the given immunogen. When compared with DeOAc-LPS, O-SP induced 18.3-fold higher stimulated peak secretion of TNF- α . Stimulation with O-SP also resulted in a 2.6-fold peak increase in IL-1 α secretion and a 3.3-fold increase in IL-6 in comparison with DeOAc-LPS stimulation.

The time course of cytokine secretion in response to the stimuli was followed. The secretion of TNF- α , IL-1 α and IL-6 was measured at different time intervals (3, 6 and 24 h) after MΦ stimulation (Figs 1 and 2). It was of interest that, although the extent of cytokine secretion in response to DeOAc-LPS was less than that induced by O-SP, the time course of secretion of the relevant cytokines was similar, with the exception of TNF- α . DeOAc-LPS- and O-SP-induced secretion of IL-1 α peaked after 3 h, followed by a substantial decrease reaching low or undetectable values 24 h after stimulation. The time course of TNF- α secretion was clearly different, with the most significant increase being observed during the first 3 h in response to DeOAc-LPS. The O-SP-stimulated peak amount of TNF- α

Fig. 1. Time-dependent cytokine secretion induced by the glycolipid moiety (DeOAc-LPS) derived from LPS detoxified by base hydrolysis. Murine peritoneal MΦs were stimulated for 3, 6 or 24 h with 12.5 – $50\text{ }\mu\text{g ml}^{-1}$ DeOAc-LPS ml^{-1} . Spontaneous (baseline) IL-6 and TNF- α secretion of unstimulated MΦs was less than 22 and $15\text{ }\mu\text{g ml}^{-1}$, respectively. The data represent means $\pm$ SEM of triplicate values. Comparisons of data groups were performed against unstimulated baseline cytokine secretion levels. Statistically significant differences: **, $P < 0.01$; ***, $P < 0.001$. NS, Not significant.

Fig. 2. Time-dependent cytokine pattern induced by O-SP derived from LPS detoxified by acid hydrolysis. Murine peritoneal M Φ s were stimulated for 3, 6 or 24 h with 12.5–50 $\mu\text{g O-SP ml}^{-1}$. Spontaneous unstimulated IL-6 and TNF- α secretion was less than 22 and 15 pg ml^{-1} , respectively. The data represent means $\pm$ SEM of triplicate values. Comparisons of data groups were performed against unstimulated baseline cytokine secretion levels. Statistically significant differences: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. NS, Not significant.

culminated 6 h after M Φ stimulation. Twenty-four hours after activation, the maximum peak levels of TNF- α were reduced by 89.1 and 90.8 %, respectively, compared with the levels observed 3 h after M Φ activation. DeOAc-LPS- and O-SP-induced IL-6 levels exhibited a time-dependent increase and remained significantly elevated ($P < 0.001$) at 24 h after activation.

The time- and concentration-dependent ROS release was also determined after 3, 6 and 24 h of M Φ activation (Fig. 3). DeOAc-LPS- and O-SP-stimulated production of ROS peaked after 3 h, and then declined. The differences between ROS liberation by the two immunogens were observed throughout the concentration range. O-SP induced a 1.11-fold higher maximal peak value compared with DeOAc-LPS. Twenty-four hours after DeOAc-LPS and O-SP activation of peritoneal M Φ s, the maximum peak levels of ROS were reduced to 57.2 and 52.4 %, respectively, compared with the levels observed 3 h after M Φ activation. The maximal values reached were detected following stimulation with 50 $\mu\text{g DeOAc-LPS}$ or O-SP ml^{-1} . The kinetics of the stable end metabolite NO_2^- was measured to determine the induction of NO. The results of the dose- and activation time-dependent release of NO_2^- are summarized in Table 1.

The results of this study indicate the capability of these two dLPS-derived structures to induce pro-inflammatory mediators in M Φ s with respect to the extent of reduction or total elimination of the original toxicity of LPS. The important question was the impact of the two different chemical detoxification approaches on the immunomodulation capacity of the two derivatives.

LPS, a strong activator of innate immunity, is frequently associated with immunological inflammation or Gram-negative septic shock. Receptor-mediated attachment of LPS results in the activation of M Φ s followed by the release of inflammatory mediators, including various pro-inflammatory ILs such as IL-1 β , IL-1 α , IL-6, IL-8 and IL-12, IFNs such as IFN- γ , and chemokines and growth factors such as MCP-1, GC-CSF and TNF- α . The cytokine triad of TNF- α , IL-1 α and IL-6 represents the main LPS-induced response. Our study of dLPS-derived substances showed the induction of only this triad (Figs 1 and 2), whilst the secretion of other cytokines and growth factors was not detected by FlowCytomix analysis. The close association between IL-1 and IL-6 levels and the severity of septic shock has been documented. Moreover, IL-1 markedly potentiates the toxic effect of TNF- α in animal experiments (Waage *et al.*, 1991). TNF- α regulates cytokine and

Fig. 3. Time- and concentration-dependent release of ROS induced by the LPS-derived saccharide moieties O-SP (a) and DeOAc-LPS (b). The data represent means $\pm$ SEM of triplicate values. Comparisons of data groups were performed against unstimulated baseline ROS expression levels using ANOVA. Statistically significant differences: **, $P < 0.01$; ***, $P < 0.001$. ns, Not significant.

chemokine gene expression in a number of cells involved in the host response to infection and initiates a cascade of cytokines associated with infection. The effective biological activities of TNF- α comprise activation of T and B lymphocytes, M Φ s and natural killer cells; fever induction; acute-phase protein release; and endothelial cell activation. The primary source for TNF- α is activated monocytes and M Φ s, especially after priming by IFN- γ . It is a key regulator of other pro-inflammatory cytokines such as IL-1 α , IL-6 and IL-8. The generalized overproduction of TNF- α during Gram-negative bacterial infections causes widespread tissue damage and represents a common cause of circulatory

shock leading to death. IL-1 α is a highly inflammatory cytokine similar to TNF- α in many aspects. It has a major influence on diverse cell populations and exerts biological effects including fever and increased synthesis of acute-phase proteins. IL-1 α is secreted by M Φ s activated by a number of different stimuli such as TNF- α and/or bacterial endotoxin.

IL-6 has usually been regarded as a pro-inflammatory cytokine induced by LPS simultaneously with TNF- α and IL-1. IL-6 is frequently exploited as a marker of systemic activation of pro-inflammatory cytokines. Although IL-6 stimulates liver secretion of acute-phase proteins, it has anti-inflammatory properties as well. IL-6 downregulates the synthesis of IL-1 and TNF- α . The ratio of IL-6 to TNF- α may be predictive with regard to chronic inflammation (Koj, 1985; Tilg *et al.*, 1994; Opal & dePalo, 2000; Dube *et al.*, 2004).

Based on our results with dLPS-derived substances (Fig. 1 and Fig. 2), the various cytokine interactions could be supposed to include IL-6-promoted downregulation of IL-1 α and TNF- α . The generation of cytokines was more pronounced with O-SP stimulation, probably due to the structural arrangement of the molecule and epitope accessibility [probably located in the core 3-deoxy-D-

Table 1. M Φ release of NO₂⁻ (mmol l⁻¹) induced by DeOAc-LPS and O-SP

Concn of stimulant (µg ml ⁻¹)	DeOAc-LPS			O-SP		
	3 h	6 h	24 h	3 h	6 h	24 h
12.5	3.44	5.27	16.95	6.74	8.37	38.97
25	17.83	18.26	40.23	3.44	7.81	—
50	10.62	121.75	38.34	3.44	34.69	33.52

manno-octulosonic acid (KDO) region, see below]. The production of ROS (Fig. 3) simultaneously with pro-inflammatory TNF- α , IL-1 α and IL-6 cytokine release demonstrated that effective M Φ immunoenhancement is more expressive with O-SP.

The reactive L-arginine-derived free radical NO is produced by NO synthase. NO is generated in M Φ s following exposure to various stimuli, such as TNF- α , IL-1 β , IFN- γ and LPS (Chi *et al.*, 2003). Cytokine- and dLPS-triggered production of NO $_2^-$ was observed with both LPS-derived moieties (Table 1). Excreted TNF- α and IL-1 might synergistically affect M Φ activity over the time course studied. NO $_2^-$ synthesis occurs at 3–6 h post-activation and progresses linearly up to 24 h (Mosser & Zhang, 2008). Our results (Table 1) are in good agreement with these observations. Both tested substances induced an increase in NO $_2^-$ values in a time- and dose-dependent manner.

Based on these results, it was concluded that the observed pro-inflammatory cytokine trigger and ROS/NO generation corresponded to M Φ activation by the O-SP and DeOAc-LPS fractions of LPS. This observation will be important for future safe and efficient immunization/vaccination with subcellular vaccines based on LPS-derived fragments. Generally, the immunomodulative properties of LPS are ascribed to the polysaccharide core antigenic moieties and the somatic O-antigen. The inner-core-derived oligosaccharides trigger mononuclear cells to produce the immunostimulating cytokine IL-1 (Lebbar *et al.*, 1986). Synthetic or natural oligosaccharides such as the core-derived heptose (Hep) II–Hep I–KDO I trisaccharide induce the release of IL-1 from human mononuclear cells (Johnson, 1994). The minimal structure required to trigger IL-1 secretion seems to be a disaccharide that contains a neutral sugar (e.g. heptose) (Rietschel *et al.*, 1996).

As predominant surface structures, O-antigens are highly immunogenic and O-specific antibodies are frequently used for serotyping, in clinical diagnosis, and for epidemiology and taxonomy. Moreover, O-antigens are involved in both the activation and inhibition of the complement cascade (Tanamoto *et al.*, 1984; Viswanathan *et al.*, 2004). The O-polysaccharide has also been associated with the cell attachment of LPS in host–parasite interactions and the induction of cytokine release.

Various reported data have indicated that the polysaccharide moiety of LPS molecules is important for its biological activity and is involved in stimulation of human monocytes (Otterlei *et al.*, 1993; Cavaillon *et al.*, 1989). KDO, which forms the link between lipid A and the polysaccharide in LPS, is a remarkably immunopotent molecule, as illustrated by LPS mutants lacking the entire polysaccharide chain except for KDO (Cavaillon, 1994). Pro-inflammatory cytokine release especially IL-1 by monocytes as a response to KDO has been documented (Lebbar *et al.*, 1986).

Our results on M Φ cytokine induction with detoxified LPS-derived immunogenic structures are in agreement

with the important role of the core KDO region in the immunomodulation process. Immunogenic O-SP isolated by acid hydrolysis of *V. cholerae* LPS has the KDO structure readily accessible in a terminal position. In contrast, the KDO structure in the second immunogen that we examined, DeOAc-LPS, is internal. The induction of pro-inflammatory cytokines and ROS/NO $_2^-$, which was more profound in the case of the O-SP moiety, may be the result of such a structural arrangement.

Novel trends in the development of an anti-cholera vaccine are directed towards dLPS-derived structures and their ability to induce a potent immunogenic effect. Subcellular vaccines represent one of the recent trends in saccharide vaccine design. Conjugation of thymus-independent saccharide moieties with a protein carrier shifts such constructs towards thymus dependency. It has been shown to be possible to make an effective immunogen based on subcellular low-molecular-mass oligosaccharides (Chernyak *et al.*, 2002; Paulovičová *et al.*, 2006). A better understanding of the structure–immunomodulating activity relationship will permit the development of selective anti-cholera subcellular vaccines.

The present investigation of dLPS-derived structures revealed the effective acceleration of ROS/NO $_2^-$ production and pro-inflammatory TNF- α , IL-1 α and IL-6 cytokine release by stimulated mouse peritoneal M Φ s. These results indicate an effective immunomodulating activity of two *V. cholerae* O1 Ogawa El Tor dLPS-derived structures and provide encouraging findings for the use of such derivatives containing the core KDO region in subcellular cholera vaccine development.

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