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OBSERVATION ON THE CHANGES IN LACTATE DEHYDROGENASE ISOENZYMES IN POST-BURN PATIENTS: SIGNIFICANCE IN RELATION TO CREATINE KINASE

OPSERVACIJE O PROMENAMA U IZOENZIMIMA LAKTAT DEHIDROGENAZE KOD PACIJENATA SA OPEKOTINAMA: ZNAČAJ U ODNOSU NA KREATIN KINAZU

Riaz Ahmad[1], Saba Qayyum[1], Absar-ul Hasnain[1], Anjum Ara[1], Arshad H. Khan[2], Mumtaz Alam[2]

[1]Section of Genetics, Department of Zoology, Faculty of Life Sciences
[2]Department of Plastic Surgery, Jawaharlal Nehru Medical College and Hospital
Aligarh Muslim University, Aligarh-202002 (UP), India

Summary: The present study deals with the quantitative assessment of lactate dehydrogenase isoenzymes in the sera of burn subjects. Efforts are also made here to show better predictive marker value of sera LDH, as a few other known protein markers like creatine kinase and myoglobin have limited analytical value in the management of thermal burns. Blood was initially collected at day-1 of admission from 29 burn and 10 healthy subjects. Further, the sampling was carried out at 2, 5, 10, 20 and 30 days of wound healing (recovery). Plasma and sera LDH isoenzymes were monitored on 7.5% polyacrylamide gel electrophoresis. Quantitative assessment of LDH isoenzymes was done from gel-scans using GelPro and Scion Imaging softwares. Sera CK levels were estimated colorimetrically using reagent kits. Our results show that quantitative changes in LDH isoenzyme were more convincing and interpretable in the sera than plasma. Sera LDH-5 isoenzyme was detected as the major contributor of total sera LDH activity, which follows a change parallel to sera CK in burn subjects. Sera LDH-5 activity also remains significantly high for up to 10 days while sera CK levels were detected elevated up to 5 days ($P<0.05$) during the recovery of patients. Therefore, the present findings strongly recommended the use of sera to assess the LDH activity and indicate better stability of sera LDH-5 than sera CK during post burn wound healing.

Keywords: biomarker, lactate dehydrogenase isoenzymes, polyacrylamide gel electrophoretic profiles, sera creatine kinase, thermal burns

Address for correspondence:

Dr. Mumtaz Alam, M.Ch.
Department of Plastic Surgery,
Jawaharlal Nehru Medical College and Hospital,
Aligarh Muslim University, Aligarh-202002 (UP), India
Phone: +919358207245
e-mail: mumtaz.jnmc@rediffmail.com

Kratak sadržaj: Predmet studije je kvantitativna procena enzima laktat dehidrogenaze u serumu pacijenata sa opekotinama. Namera je bila da se prikaže bolja prediktivna vrednost markera LDH iz seruma, pošto nekoliko ostalih poznatih proteinskih markera poput kreatin kinaze i mioglobina imaju ograničenu analitičku vrednost u zbrinjavanju termičkih opekotina. Krv je uzeta prvog dana po prijemu od 29 pacijenata sa opekotinama i 10 zdravih subjekata. Zatim su uzimani uzorci posle 2, 5, 10, 20 i 30 dana zaceljivanja (oporavka). Izoenzimi LDH u plazmi i serumu praćeni su tokom elektroforeze na 7,5% poliakrilamidnom gelu. Kvantitativna procena izoenzima LDH izvršena je putem gel-skenova pomoću softvera *GelPro* i *Scion Imaging*. Nivoi CK u serumu određeni su kolorimetrijski korišćenjem komercijalnih reagenasa. Rezultati pokazuju da su kvantitativne promene u izoenzimima LDH bile ubedljivije i lakše za tumačenje u serumu nego u plazmi. Uočeno je da je izoenzim LDH-5 iz seruma glavni činilac ukupne aktivnosti LDH u serumu, koji prati paralelnu promenu CK u serumu pacijenata sa opekotinama. Takođe, aktivnost LDH-5 u serumu ostaje značajno povišena i do 10. dana, dok su nivoi CK u serumu povišeni do 5. dana ($P<0,05$) oporavka. Stoga naši rezultati ukazuju na bolju stabilnost LDH-5 u serumu nego CK u serumu tokom procesa zaceljivanja opekotina, te se preporučuje upotreba seruma za procenjivanje aktivnosti LDH.

Ključne reči: biomarker, izoenzimi laktat dehidrogenaze, elektroforetski profili poliakrilamidnog gela, kreatin kinaza u serumu, termičke opekotine

Introduction

Proteins and enzymes or their multiple forms are fairly reliable markers to assess the type and extent of damage to tissues under clinical conditions. Among them, lactate dehydrogenase (LDH, EC. 1.1.1.27.) is

Abbreviations: lactate dehydrogenase (LDH); creatine kinase (CK); polyacrylamide gel electrophoresis (PAGE).

a sensitive marker that has found extensive use in diagnostics, either as the total activity or as isoenzymes (1–5).

LDH is a tetrameric enzyme that during anaerobic glycolysis catalyzes the inter-conversion of lactate to pyruvate using either NAD or NADH as cofactors. Following electrophoresis, human LDH resolves into five isoenzyme bands. Two of them, LDH-1 and LDH-5 are homotetramers of heart predominant H and muscle specific M subunits, and form random heterotetrameric combinations LDH-2, LDH-3 and LDH-4, respectively (1). The activity levels of individual isoenzyme bands are determined by the physiological function and metabolic or clinical state of affected tissue. Tissue-specific normal isoenzyme profiles of humans are therefore found disturbed in a number of clinical conditions and thus carry diagnostic value. For instance, elevated serum LDH levels are reported in myocardial infarction, hepatitis, muscle diseases and effusions, megaloblastic and haemolytic anaemias, leukemia, lymphoma, infectious mononucleosis, and both necrotic as well as obstructive jaundice (2–6). In certain pathological states, besides sera (7) and plasma (8–11), LDH isoenzyme levels also vary in blood platelets (12–14). Only a few published reports have dealt with LDH activity leakage in the sera of burn patients (15–16). Despite the vast body of published evidence available on other clinical conditions, it is intriguing that the comparative value of LDH isoenzymes in burn subjects has not been fully evaluated.

For assessing degrees of burns and per cent surface area of the patient's body, myoglobin contents and sera creatine kinase (sCK) activity have been established as markers (17). Myoglobin has better value specifically in the management of electrical burns, while sCK may also be taken as a marker in thermal burns. Since previously spillage of LDH in the sera has been correlated with the tissue damage (5, 15–16), it is however reasonable to assume that similar to sCK, LDH should also leak out into serum when muscle or related tissue in burn patients is damaged. Here we have made an attempt to address LDH related issues to propose its better marker value in burn subjects, and carried out: 1) a comparison in the plasma and sera LDH changes to work out the suitability of serum in monitoring post-burn LDH adjustments, since the presence of LDH-rich platelets in plasma may interfere with the activity levels; and 2) during post-burn recovery, changes in sera LDH (sLDH) isoenzyme levels were compared with the course taken by sera creatine kinase (sCK) to sort out the relationship that exists between the two. So far as we know this is the first conclusive study that demonstrates a correlation between a rise in sLDH activity and sCK in thermal burns along with the adjustments within individual LDH tetramers leading to a rise in the total LDH activity in post-burn subjects.

Materials and Methods

Sample Collection

The patients were approached in the Burn Ward of the Department of Plastic Surgery, Jawaharlal Nehru Medical College and Hospital (JNMCH), AMU Aligarh. None of them had any pre-burn injuries nor was on any other treatment regimen. Patients with total burn surface area (TBSA) of 30% (18) were included in the present study. Blood was taken from 29 burn subjects by 2 mL sterilized syringe. Out of it, 1 mL was mixed with sodium heparin for plasma whereas the remaining was kept as such in sterilized Eppendorff tubes to ooze out the sera. Plasma and sera from 10 healthy individuals served as control. Healthy subjects were selected with no record of pre-burn injury or treatment course. Blood taken at day-1 of admission of the patients to the hospital was used to analyze the plasma and sera LDH levels. In samples taken at day 2, 5, 10, 20 and 30 during post- burn recovery of the patients, LDH activity was measured in the sera only. The study was carried out up to 30 days before all the patients recovered fully and were discharged from the hospital. Experiments were carried out in compliance with the regulations laid by the University Ethical Committee.

Protein Estimation

Clear plasma was obtained following the centrifugation of sodium-heparinized plasma at a speed of 3,500 rpm (REMI; C-24BL) for 5 min. Protein concentration in plasma and sera samples of burn and healthy subjects was measured on 10UV-Visible Spectrophotometer (Thermospectronic, USA) using the standard protocol of Lowry et al. (19). Equal amounts of fresh plasma and sera (~20 μL) of burn subjects were loaded onto polyacrylamide gels for LDH analysis. Activity of sera creatine kinase (sCK) in burn and healthy individuals was estimated colorimetrically using reagent kits (Ranbaxy Laboratories Ltd., Mumbai). Each experiment was performed in triplicate and, following quantitation by densitometry, their mean ± standard deviation was calculated.

Polyacrylamide Gel Electrophoresis (PAGE) and Visualization of LDH Isoenzyme Bands

PAGE was performed on 7.5% polyacrylamide vertical slab gels (10×9.4×0.1 cm) as described previously (20). Runs were initially made in 1× upper gel buffer (Tris-HCl; pH, 6.8) until the samples entered the stacking or lower separating gel and were then replaced with Tris-glycine as the running buffer. It improved the resolution and sharpness of bands by avoiding distortions and smearing effects.

LDH bands (isoenzymes) were visualized by incubating the gels in a specific stain containing 5 mL of 1 mol/L L-lactate as substrate, 3.77×10^{-3} mol/L

β-NAD, 3.26 × 10^{-4} mol/L phenazine methosulphate as intermediate and 1.83 × 10^{-3} mol/L nitro blue tetrazolium as the hydrogen acceptor in 100 mL of 50 mmol/L Tris-HCl buffer (pH, 7.5). Gels were incubated in reaction mixture at 37 °C for 30 min to stain and visualize the LDH isoenzyme bands.

Densitometry and Statistical Analysis

Densitometric analysis of gel-scans was made with Scion Imaging software (Beta release-4, Scion Corporation) while the quantitative estimate of each PAGE lane was performed using GelPro software program (Media Cybernetics, USA). The values of LDH isoenzymes and CK activities in plasma and sera were presented as mean ± SD (n=3) for further data analysis. Student's *t*-test was applied and the significant differences in the mean values of enzyme (or isoenzymes) activities in plasma/sera of burn subjects with respect to their levels in healthy individuals were noted. Numerical differences obtained were considered statistically significant at $P < 0.05$.

Results

Typical lactate dehydrogenase polyacrylamide gel electrophoretic (LDH-PAGE) profiles showed differing intensities of isoenzymes in post-burn as well as healthy subjects (*Table I*). Densitometric analysis of LDH gel scans of burn and healthy subjects revealed five bands in the plasma as well as in sera. The representative lane of patients sera LDH profile is also displayed along with the densitogram (*Figure 1*). Quantitative estimates of LDH isoenzymes in plasma and sera at day-1 showed significant differences ($P<0.05$), however sera LDH profiles were found more convincing (*Table I*). Plasma LDH (pLDH) profiles were similar in any case (LDH-3>-4>-2>-5>-1) and demonstrated nearly eight times higher activity of LDH compared to sera.

Noticeable differences were displayed by the sera LDH isoenzymes in burn and healthy subjects at the time of admission of the patient i.e. at day-1. In the patients, detectable differences and quantitative adjustments were displayed specifically by sLDH-5 and sLDH-4. Sera LDH-5 showed a significant increase from 35.92±1.55 to 37.72±1.21 ($P<0.05$), whereas sLDH-4 demonstrated a decline from 37.34±1.61 to 33.36±1.86 (*Table I*). Overall at day-1, pattern of relative intensities followed a trend: LDH-5>-4>-3>-1>-2 in the sera of patients, while in healthy individuals it was LDH-4>-5>-3>-1>-2. Extended sera

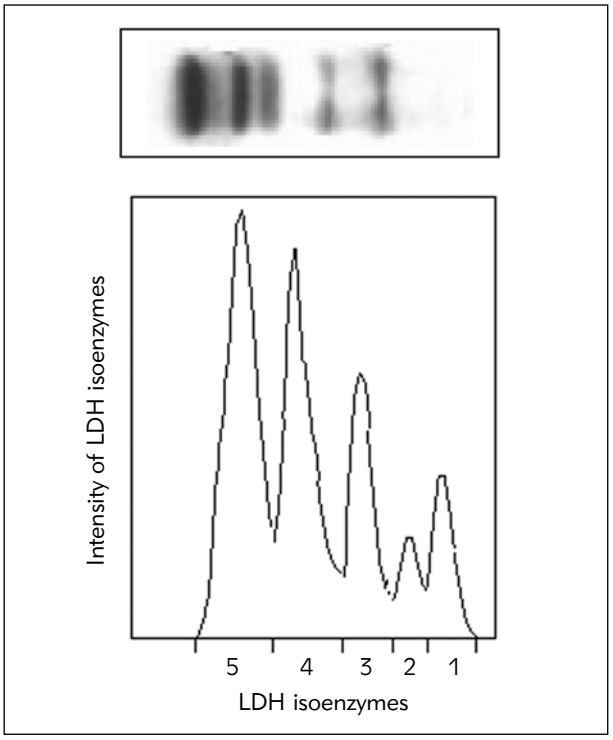

Figure 1 Densitometric tracing of LDH gel scans (lane shown) in sera sample of burn subjects. Individual peaks show the existence of LDH isoenzymes.

Table I Percent LDH isoenzyme activity (arbitrary units) in the plasma and sera of healthy individuals (control) and burn subjects at day-1 post-burn. Values are Mean ± SD of individual LDH-PAGE lanes of electrophoretic runs made under identical experimental conditions.

	Plasma			Sera		
LDH isoenzymes	Healthy subjects (N=10)	Burn subjects (N=29)	LDH isoenzyme level	Healthy subjects (N=10)	Burn subjects (N=29)	LDH isoenzyme level
LDH-5	6.25±0.041	8.60±0.09	Increase	35.92±1.55	37.72±1.21	Increase
LDH-4	39.31±1.15	37.63±1.25	Decrease	37.34±1.61	33.36±1.86	Decrease
LDH-3	41.05±1.32	44.31±1.67	Increase	20.41±1.09	22.82±1.18	Increase
LDH-2	12.89±1.06	9.21±0.5	Decrease	2.91±0.061	1.93±0.040	Decrease
LDH-1	0.50±0.002	0.25±0.0015	Decrease	3.42±0.082	4.17±0.068	Increase

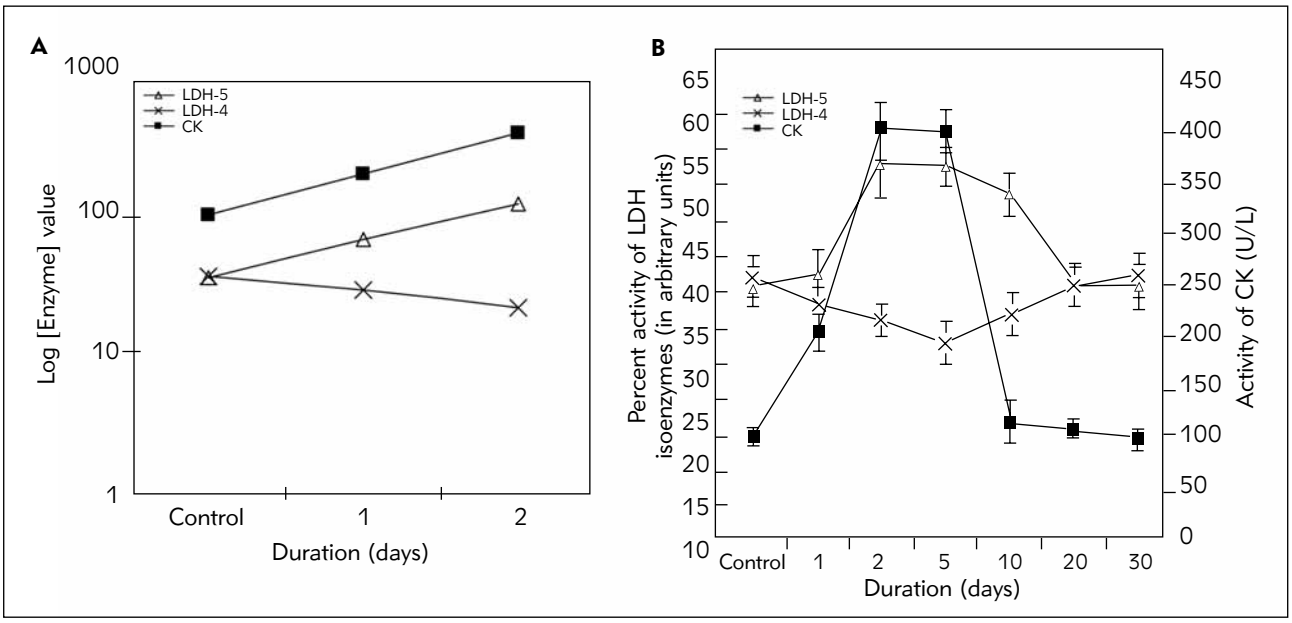

Figure 2 A. Relationship between the initial changes in log values of sera enzymes activities (LDH-5, -4, and CK) and the duration of recovery in burn subjects. B. Course of percent change in the activities of LDH-5 and -4 and, CK in the sera of burn subjects (N=29). Values are plotted as mean ± SD.

sampling of burn subjects at day 2, 5, 10, 20 and 30 post-burn recovery revealed significant shift of LDH isoenzyme activities towards control values.

Comparison of sLDH with sera creatine kinase (sCK) activity demonstrated a parallel relationship between sLDH-5 and total sCK levels. Log [enzyme] values obtained for sLDH-5, -4 and sCK activities in the initial two days were also observed to follow a similar trend (*Figure 2A*). The values of sLDH-5 and sCK were the highest on day-2 and found 53.12 and 402.3 respectively, whereas sLDH-4 showed the least value of 28.01 on day-5 post-burn. It is interesting to notice that the elevated activity of sLDH-5 lasts up to day 10 compared with sCK activity where it is detected up to day 5 of post-burn recovery (*Figure 2B*). Enzyme screening during wound healing (recovery) of patients also revealed a similar course of change in sCK and total sLDH activity with significantly higher values of sLDH in burn subjects (*Figure 3*).

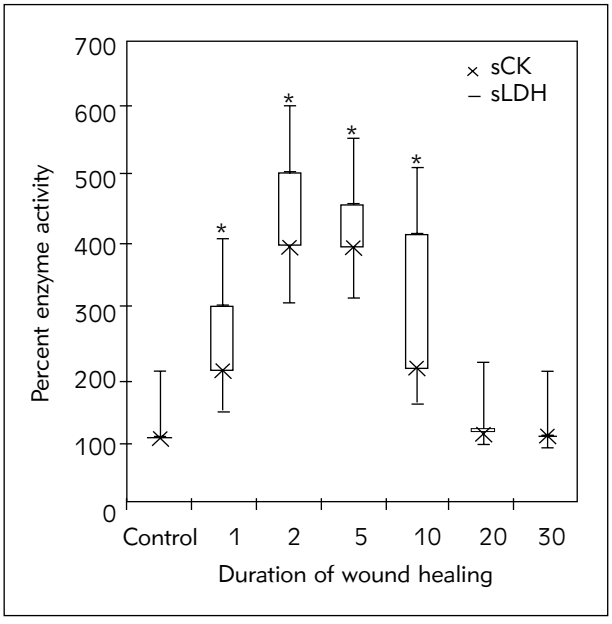

Figure 3 Change in percent activities of CK and LDH enzymes with the duration of wound healing (recovery) in sera samples of burn subjects. Vertical rectangles demonstrate differences in the mean value of the two enzymes. Asterisk (*) shows significant difference in enzyme activities ($P < 0.05$).

Discussion

Although wound healing in burns is so obvious that it hardly needs any enzymatic diagnostic marker in cases with 30% thermal burns, the need for a marker appears when it is an objective assessment rather subjective for treatment. In this way the present study becomes significant in documenting the lactate dehydrogenase isoenzymes in the form of data which may help in sorting out medico-legal problems. We have therefore tested the extensively used clinical marker lactate dehydrogenase isoenzymes data to demonstrate their compatibility with serum creatine kinase activity during post-burn healing. Sera CK activity has been documented as the standard marker of post-burn injury (17). Using both total LDH (tLDH)

activity assay and polyacrylamide gel electrophoretic (PAGE) profiles, we have worked out why serum and not plasma is a suitable source for this follow-up. PAGE profiles of plasma LDH (pLDH) and sera LDH (sLDH) isoenzymes have been employed to work out the dynamics of LDH homo- and heterotetramer adjustments and identified the highest contributors to tLDH activity.

Though the marker value of pLDH isoenzyme levels has been emphasized in certain allergies or inflammations (21–22), in our study neither total pLDH nor plasma isoenzyme profiles had the attributes of suitable markers for monitoring post-burn repair. Whereas a four-fold elevation in total pLDH activity as compared to sLDH has previously been reported (8, 11), our analyses registered an about eight-fold rise in tLDH activity in the plasma of burn subjects. It has already been demonstrated that pLDH level rises due to hemolysis caused by thermal burns (23). The main contributor to this increase in pLDH is the leakage of isoenzymes from platelets which are reported rich in LDH-2, -3 and -4 (11, 12, 24). That may be the reason of a many-fold rise in pLDH levels and that of similar ranking between plasma LDH-PAGE profiles of burn subjects and the healthy individuals which was observed as LDH-3>-4>-2>-5>-1 (*Table I*). So far as the ranking in healthy individuals was concerned, it was in conformity with reports published earlier (13, 25).

The results of this study on the relative intensities of sera LDH (sLDH) isoenzyme PAGE profiles in controls were in agreement with the previous reports (21, 26). Our findings on the sera of burn patients were, however, different from those of Liu et al. (16) who reported 6 bands in the serum of a single patient with 20% burn. PAGE profiles under our experimental conditions consistently revealed 5 sLDH isoenzyme bands in patients with 30% thermal burns (*Figure 1*). The most obvious reason for this discrepancy is serum analysis of a single patient and the degree of burn (16), though the differences in analytical procedures used here and those employed in the above-cited report cannot be entirely ruled out. However, the reliability of our data lies in the higher number of patients whose sera were analyzed.

Sera LDH isoenzymes represent their leakage from some damaged cell (21–22, 26). Hence, a rise in specific isoenzymes simply reflects the tissue source of the damaged cells, irrespective of the damage which is either caused by burns, inflammation or any other mechanism (16, 21–26). In burn subjects the most affected tissue is muscle and also the most probable source of isoenzymes spillage into the blood stream. It has been proposed that some infections or inflammation damages the muscles thereby increasing release of granulocytes or polymorphonuclear leukocytes at the site of inflammation (5, 21–22). Therefore, it is likely that similar to other pathological states of sLDH elevation, the higher levels of granulocytes or polymorphonuclear leukocytes might have contributed to the significant increase in sera LDH-5 from 35.92 ± 1.55 to 37.72 ± 1.21 ($P < 0.05$), as observed in burn subjects during the present study.

During initial post-burn recovery, sLDH-5 follows a parallel trend of increase with sCK. Thus, the course of rise in sLDH-5 activity is similar to the elevation pattern of sCK activity (*Figure 2A*). More importantly, in comparison with sCK activity, it has a clear advantage of being there as a stable form that can be monitored for 10 days. Since the increase in sLDH-5 isoenzyme persists longer than sCK, it may also have a role in the post-burn healing mechanism as a pyruvate oxidant (27). Similarly, the repair process of damaged tissue might have involvement by sLDH-3 which also shows an increase in its activity on the first day post-burn (*Table I*). In contrast to it, sLDH-4 followed an opposite kinetics suggesting the activity rise in sLDH-5 isoenzyme may be at the expense of sLDH-4 during this process (*Figure 2B*). Our results also demonstrate that similar to sCK, the total sLDH level also rises in burn subjects and remains in a significantly detectable amount till day 10 post-burn. Therefore, it may be proposed that LDH-5 is the major contributor to the activity of total LDH in serum and, as the whole enzyme system (LDH) or isoenzyme (LDH-5) their kinetics are comparable with sCK (*Figure 3*).

Therefore, while recommending investigations which are entirely based on the quantitation of LDH or its isoenzymes, it is advisable to prefer the serum as a source of diagnostic LDH analyses for its accurate estimation. It may also be suggested that sera LDH-5 is a marker of greater value than sCK due to its better reproducibility and stability up to 10 days during post-burn recovery. Lesser stability of sCK levels in assessing muscle damage has already been demonstrated in cases of electrical injuries (28).

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