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Modulation of macrophage structure and function by low level He–Ne laser irradiation

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Studies have shown that He–Ne laser irradiation can affect the biological functions of macrophages. The aim of the present study was to investigate the effect of He–Ne laser irradiation on the various functional parameters of macrophages and look for possible correlations in the effects to understand the mechanisms involved. Mice peritoneal macrophages were irradiated with a He–Ne laser (632.8 nm, ~10 W m^{-2}) at energy densities ranging from 100 to 600 J m^{-2} and the activities of lysozyme and cathepsin, phagocytosis, and cell spreading (markers of cell activation), as well as changes in NAD(P)H autofluorescence, were monitored. He–Ne laser irradiation was observed to lead to significant changes in all the parameters investigated. While lysozyme activity and spreading of the peripheral membrane were found to increase with the irradiation dose over the dose range investigated, the phagocytotic activity of macrophages, the activity of cathepsin, the observed decease in cell membrane fluidity and the observed increase in NAD(P)H level showed a peak at 200 J m^{-2}. Possible reasons for and the significance of the observed correlations are discussed.

Introduction

There are several reports suggesting that low level laser irradiation can influence wound healing, cell-mediated hypersensitivity reactions and inflammatory processes,[1–4] However, the mechanism of laser-induced immunomodulatory effects is not clear. To understand this at the cellular level, studies have been made on immunocompetent cells such as lymphocytes and macrophages. Studies with lymphocytes show that He–Ne laser irradiation can lead to an increase in immunoglobulin secretion,[5] cytokine production,[6] phagocytotic activity[7] and leukocyte priming.[8] Studies with macrophages show an increase in the enzyme activity of lysozyme and acid phosphatase,[9] stimulation of the release of growth factors,[10] and an increase in phagocytotic activity.[5] Although some of these effects have been attributed to an increase in the permeability of cell membranes to calcium ions,[11] the mechanism of red light-induced effects on macrophages is not clearly understood. In particular, information on cellular signal transduction and processing in response to laser exposure is lacking.

In order to gain further information, we have investigated the effect of He–Ne laser irradiation on the enzyme activity, phagocytotic activity, cell morphology and membrane fluidity of mice peritoneal macrophages. Recent reports suggest that modulations in intracellular NAD(P)H concentrations may be linked to downstream cell functions in neutrophils and macrophages exposed to external stimuli.[12–14] We have therefore also measured the autofluorescence of pyridine nucleotides [NAD(P)H] to investigate the involvement of metabolic signaling pathways in laser-induced effects.

Materials and methods

Isolation of peritoneal macrophages

Male Swiss albino mice, 3–4 months old, were used for isolation of peritoneal macrophages. Housed in plastic cages under controlled environmental conditions with a 12 h light/dark cycle, the mice had free access to both water and standard food. The mice were injected intraperitoneally with 1 ml 3% starch, 3 days before the collection of peritoneal exudate cells (PECs). PECs were harvested by peritoneal lavage with 8–10 ml cold 0.01 M phosphate-buffered saline (PBS) at pH 7.2. The peritoneal fluid was aspirated and the cell suspension was centrifuged at 300*g* for 30 s. The cells were resuspended in cold PBS containing 10 mM glucose at a cell concentration of 4–6 × 10^4 cells ml^{-1}. The cell viability was measured using the Trypan Blue exclusion method. The cell suspension was stored on ice until it was required.

Irradiation procedure

1 ml cell suspension in PBS was taken into a quartz cuvette (10 mm path length, 3 ml capacity) and irradiated in the dark at room temperature (27 °C) under continuous stirring. The irradiation was performed using a He–Ne laser (632.8 nm, ~25 mW, Russian made) with a beam area expanded to ~2.3 cm^2 to cover the whole cell suspension. The laser power was measured using a Scientech-372 power meter and adjusted to ~2.3 mW using an appropriate neutral density filter to give a power density of 10 W m^{-2} at the front surface of the cuvette. The irradiation dose was varied from 100 to 600 J m^{-2} by changing the irradiation time from 10–60 s. Following irradiation, both unirradiated and irradiated cells were incubated for 30 min (37 °C, 5% CO_2 in humidified air) and then used for various measurements, as described below.

Measurement of enzyme activities

Lysozyme activity was determined by the spectrophotometric method, as described in ref. 15. Briefly, the peritoneal cell suspension was added to a reaction mixture containing wild-type *Escherichia coli* K12 (AB1157) cells and 1% Triton X-100 in PBS, and the change in absorbance at 450 nm was monitored for 10 min. The absorbance of the reaction mixture at 0 min was ~0.6. Lysozyme activity is expressed in units min^{-1}. The change in absorbance of 0.01 at 450 nm was defined as one unit.

Cathepsin activity was measured spectrophotometrically following the method described in ref. 16. The enzyme was released from the cells by sonication treatment for 5 min under cold conditions. After centrifuging at 600*g* for 5 min, the supernatant was collected and used for the assay of cathepsin activity. The reaction was started by adding the supernatant to a solution containing 10% bovine serum albumin in 0.05 M phosphate buffer at pH 3.5. After incubation at 37 °C for 60 min, the reaction was stopped by adding 5% trichloroacetic acid solution. The contents were allowed to stand at room

temperature for 10 min and then centrifuged at 6000*g* for 10 min. The supernatant was then reacted with Folin–Cioalteau reagent and the absorbance of the resultant chromagen was read at 600 nm using a Shimadzu UV3101 spectrophotometer. Cathepsin activity is expressed as units mg(protein)$^{-1}$ min^{-1}. The absorbance of 0.01 at 600 nm was defined as one unit.

Measurement of phagocytotic activity

Phagocytotic activity was measured using a fluorochrome assay[17] using baker's yeast (*Saccharomyces cerevisiea*) purchased from the local market. The yeast cells were opsonized by suspending them in 10% fetal bovine serum in PBS followed by incubation at 37 °C for 30 min. Macrophages were allowed to adhere to glass coverslips for 30 min at 37 °C in a humidified (approx. 98%) atmosphere containing 5% CO_2. Subsequently, the non-adhered cells were removed by washing the coverslips with MEM medium and a suspension of opsonized yeast cells in MEM medium was laid over the coverslips. The coverslips were placed onto saline-moistened tissue paper in a petri dish and incubated for 90 min at 37 °C in a humidified CO_2 incubator. After incubation, the coverslips were rinsed with PBS and stained with Acridine Orange. The coverslips were counterstained with Crystal Violet, mounted onto larger coverslips and sealed with nail polish. The coverslips were examined under a fluorescence microscope (Zeiss Axiovert 135) with UV excitation. The number of dead yeast cells in 100 macrophages was counted. Phagocytosis is expressed as the number of red (dead) intracellular yeast cells per macrophage.

Cell surface morphology

Following irradiation, both control and irradiated cells were allowed to adhere to the glass slide for 30 min at 37 °C in 5% CO_2 and humidified atmosphere. The non-adherent cells were removed by washing with PBS. The slides were washed finally with 50% methanol and stained with a 50 μg ml^{-1} solution of a cell surface protein-SH-specific dye, 5-(iodoacetamido)-fluorescein (5-IAF; Molecular Probes Inc.). The excess dye was removed by rinsing the slides in PBS. The slides were viewed using a Zeiss Axiovert135 TV inverted microscope equipped for epi-illumination. The apple-green fluorescence of 5-IAF was viewed using a blue excitation fluorescence filter set. The fluorescence and phase contrast images were photographed on Nova black and white film (100 ASA) using a Zeiss MC80 camera equipped with an automatic exposure controller.

Measurement of membrane fluidity

Cell membrane fluidity was measured by the fluorescence polarization technique using the fluorescence membrane probe 8-anilino-1-naphthalenesulfonic acid (ANS; Sigma), as described in ref. 18. The cells were labeled with ANS by incubating the cells in PBS containing 1.0 mM ANS at 4 °C for 30 min in the dark. The cells were then centrifuged at 300*g* for 30 s. The cells were washed with cold PBS to remove the unbound probe. After further centrifuging, the cells were finally re-suspended in PBS and used for the experiment.

Fluorescence anisotropy was measured on a SPEX Fluorolog-2 spectrofluorometer. The cell suspension was excited at 380 nm and emitted fluorescence was measured at 480 nm. Steady-state fluorescence anisotropy values were obtained by simultaneous measurement of intensity components I_{vv} and I_{vh}. The vv and vh indices stand for the parallel (both vertical) and perpendicular (vertical excitation, horizontal emission) positions of the excitation and emission polarizers, respectively. A correction factor for the unequal transmission of horizontally and vertically polarized intensity components by the optical elements ($G = I_{hv}/I_{hh}$) was also determined and used in calculating the emission anisotropy as follows:

$$r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$$

Measurement of NAD(P)H fluorescence

Autofluorescence of pyridine nucleotides [NAD(P)H] was monitored on a SPEX Fluorolog-2 spectrofluorometer. The fluorescence spectrum of the cell suspension was recorded from 400–600 nm using 340 nm excitation. The excitation and emission slit were set at 1.0 mm with bandwidths of 3.7 and 1.7 nm, respectively. Following laser irradiation, the cell suspension was transferred to a quartz cuvette and spectra were recorded at times after irradiation of 0–16 min at 30 s time intervals. Between each spectral scan, the suspension was gently stirred to avoid any settling of cells. Spectra of a cell sample which had not been irradiated with the He–Ne laser were also recorded as a control.

Statistical analysis

Data are plotted as the mean ± standard deviation of at least three independent experiments. The results were statistically analyzed using Student's *t*-test for unpaired groups.

Results

Changes in macrophage activity

In Fig. 1, the measured changes in the activities of lysozyme and cathepsin (markers of macrophage stimulation) following He–Ne laser irradiation of macrophages at doses ranging from 100–600 J m^{-2} are shown. He–Ne laser irradiation was found to lead to an increase in both lysozyme and cathepsin activity [Fig. 1(a) and (b)]. While lysozyme activity was observed to

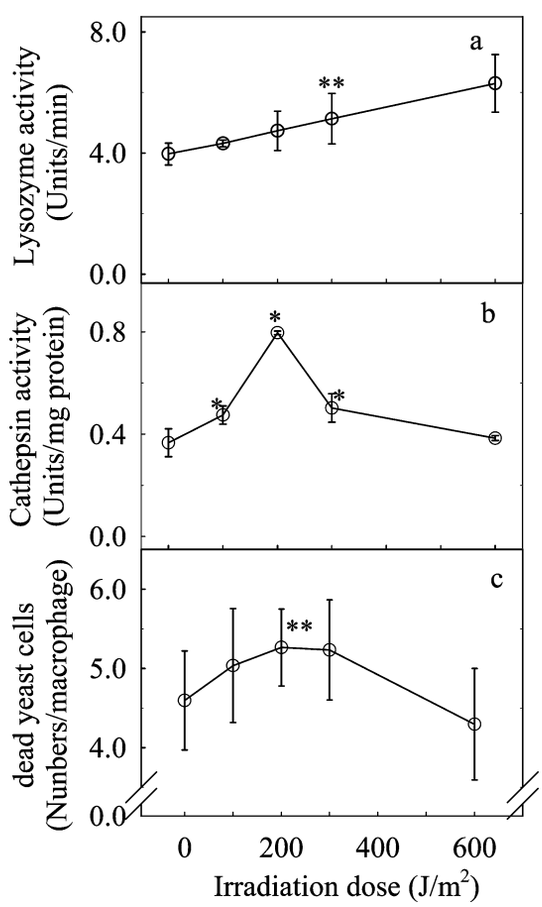

Fig. 1 Changes in lysozyme (a), cathepsin (b) and phagocytotic (c) activity of macrophages after various doses of He–Ne laser irradiation (for details, see Materials and methods section). Error bars represent the standard deviation of the mean, n = 3–4. (*) $p < 0.01$, (**) $p < 0.05$.

increase with increasing irradiation dosage over the dose range investigated, cathepsin activity was found to peak at a dose of 200 J m^{-2}.

We also monitored phagocytic activity to find out whether He–Ne laser irradiation has any influence on the cytotoxic response of macrophages. We observed that there was only a slight increase in macrophage phagocytotic activity at an irradiation dose of 200 J m^{-2} [Fig. 1(c)].

Changes in cell morphology

Macrophage spreading has been considered as a marker of cellular activation and differentiation.[19] Several stimuli, such as phorbol esters, calcium ionophor bleomycin, corticosteroids and non-steroidal anti-inflammatory drugs, *etc*., have been shown to induce macrophage spreading.[19] We therefore investigated the effect of He–Ne laser irradiation on the morphology of macrophages. Photomicrographs of cells exposed to various doses of laser irradiation are shown in Fig. 2. Cells irradiated with He–Ne laser light show an increase in cell size as compared to unirradiated cells [Fig. 2(b)–(d)]. The unirradiated cells appear round [Fig. 2(a) and (e)], whereas the irradiated cells display spreading of the peripheral plasma membrane [Fig. 2(f)–(h)].

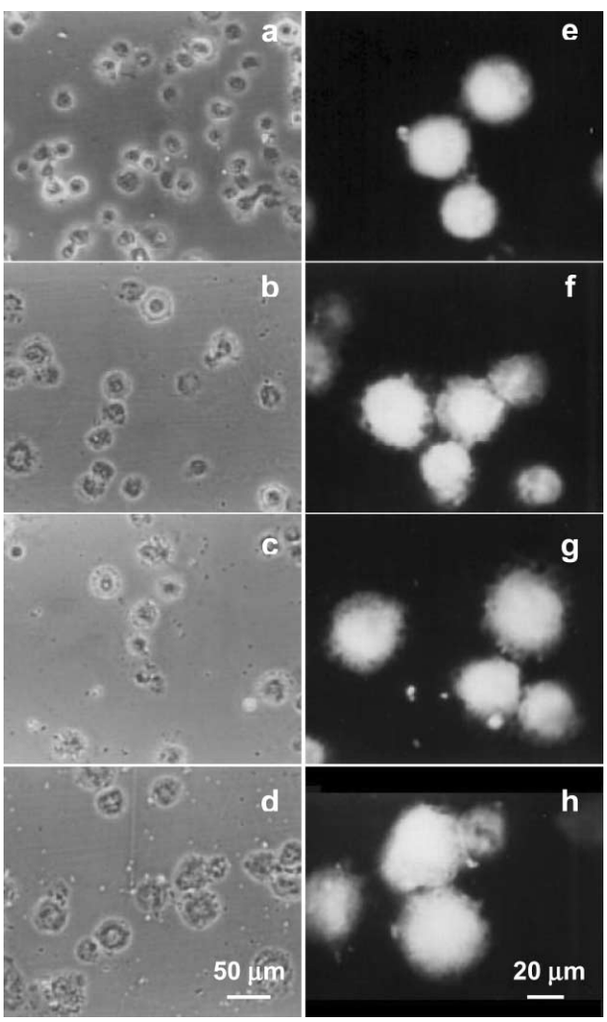

Fig. 2 Photomicrographs of macrophages after various doses of He–Ne laser irradiation: (a–d) phase contrast (magnification 40×) and (e–h) fluorescence (magnification 100×) images for the control (a, e) and 100 (b, f), 300 (c, g), and 600 J m^{-2} (d, h) irradiated samples.

Changes in membrane fluidity

The macrophage plasma membrane is known to have a regulatory role in induction of various macrophage functions such as cell adhesion, cell spreading, endocytosis, phagocytosis, *etc*. These are brought about by a change in lateral mobility of various regulatory proteins through changes in plasma membrane fluidity.[20,21] Possible changes in cell membrane fluidity following He–Ne laser irradiation were therefore monitored by the fluorescence polarization technique using ANS. The fluorescence of ANS is sensitive to the polarity and fluidity of the membrane aqueous interface.[22] Addition of ANS to macrophage cell suspension resulted in an increase in fluorescence with a large blue shift (data not shown), which indicates binding of ANS to the hydrophobic environment in the protein matrix region of the membrane.[23] He–Ne laser irradiation was observed to lead to an increase in the fluorescence anisotropy of the membrane-bound ANS and the magnitude of the effect reached a maximum when the irradiation dose was 200 J m^{-2} (Fig. 3). An increase in fluorescence anisotropy indicates a decrease in cell membrane fluidity in the regions probed by ANS. The measurement of cell membrane integrity using Trypan Blue revealed that He–Ne laser irradiation at the doses used has no adverse effects on the macrophages (data not shown).

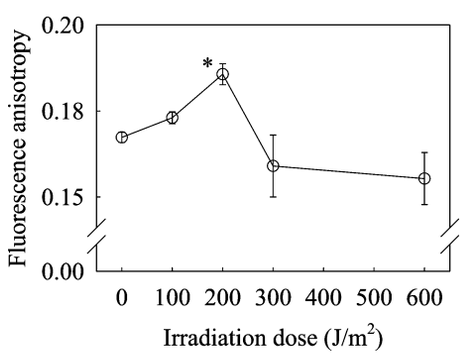

Fig. 3 Changes in fluorescence anisotropy of membrane-bound ANS following various doses of He–Ne laser irradiation (for details, see Materials and methods section). Error bars represent the standard deviation of the mean, $n = 4$. (*) $p < 0.01$.

Changes in intracellular NAD(P)H concentration

Several stimuli have been shown to modulate intracellular NAD(P)H concentration, associated with signaling events in leukocytes and macrophages.[12–14] The effect of He–Ne laser irradiation on macrophage NAD(P)H autofluorescence was therefore also monitored. 3D plots of the changes in the fluorescence spectra of the cell suspension recorded at 30 sec time interval from 0–16 min after He–Ne laser irradiation are shown in Fig. 4. NAD(P)H fluorescence intensity of control cells can be seen to remain roughly stable over this time interval whereas, the cells irradiated with He–Ne laser show a dose dependent modulation of NAD(P)H fluorescence intensity. The modulation of NAD(P)H autofluorescence intensity was most pronounced at dose of 200 J m^{-2}.

Discussion

Macrophages constitute a primary line of defense against infections playing a pivotal role in the induction, regulation and expression of immune response by acting as phagocytic, microbicidal and tumoricidal effector cells. Therefore, a good understanding of the effect of red light on macrophages may help in the understanding of the observed therapeutic efficacy of red light in the treatment of wounds,[24] rheumatoid arthritis[25] and immune system disorders,[26] *etc*. Motivated by this requirement, we have investigated the effect of He–Ne laser irradiation on several parameters in macrophages, *i.e.* enzymatic and phagocytotic activity, cell morphology, membrane fluidity and intracellular NAD(P)H concentration.

In qualitative agreement with previous studies,[5,9] He–Ne laser irradiation was observed to lead to an increase in the

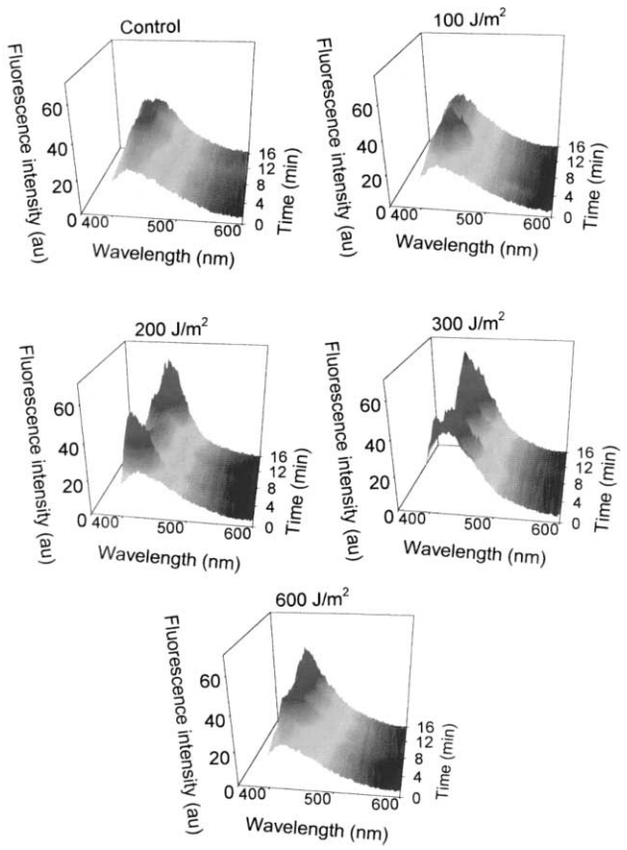

Fig. 4 3D graph showing changes in NAD(P)H autofluorescence of macrophage cell suspension in control and cells irradiated with He–Ne laser at 100, 200, 300 and 600 J m^{-2}. A total of 8 spectra were recorded from 0–16 min after irradiation, with 30 s intervals between each spectral scan, during which time the sample was stirred. For details, see Materials and methods section.

phagocytic activity of macrophages and also in the activity of lysozyme and cathepsin. However, a significant difference was observed in the dose dependence of the activity of the two enzymes. Whereas lysozyme activity was found to monotonously increase over the irradiation dose used for the experiments, the activity of cathepsin and the phagocytic activity peaked at a dose of 200 J m^{-2}. The reason for the difference in the He–Ne laser irradiation dose response for the two enzymes is not clear. It is, however, pertinent to note that while lysozyme is synthesized and secreted continuously by macrophages, even in the absence of a stimulus, the synthesis and release of cathepsin occurs only upon appropriate stimulation.[27] The two enzymes are, therefore, expected to be regulated by different cellular cytotoxic mechanisms. Thus, it appears that He–Ne laser irradiation influences these mechanisms differently.

An interesting observation is the existence of a correlation between the increase in lysozyme activity and cell spreading. Both an increase in lysozyme activity and a concomitant increase in cell spreading are involved in the endocytotic activity of macrophages. Lysozyme is responsible for digestion of extracellular pathogens and extension of the cell membrane ensures that these are engulfed by macrophages through endocytosis.[28]

Another interesting correlation was observed between the cathepsin activity, the decrease in membrane fluidity and the increase in intracellular NAD(P)H concentration [Fig. 1(b), 3 and 4]. Several reports suggest that intracellular NAD(P)H plays a significant role in cellular signal transduction and processing in the response of leukocytes to immune stimuli.[12–14] Studies have shown that intracellular NAD(P)H modulations induced by external stimuli are linked to intracellular calcium changes and downstream cell functions.[13,29] Thus, from the observed correlation, it can be speculated that the He–Ne laser irradiation-induced increase in intracellular NAD(P)H concentration leads to metabolic signaling for macrophages, leading to the observed changes in cathepsin activity and membrane fluidity.

It is pertinent to note here that because intracellular NAD(P)H is a sensitive index of mitochondrial activity,[30] the observed increase in intracellular NAD(P)H concentration may arise due to stimulation of mitochondrial activity by He–Ne laser irradiation, as has been reported by several investigators.[31–33] An increase in intracellular NAD(P)H concentration may lead to an increase in reactive oxygen species (ROS) generation by activating NAD(P)H oxidase, a plasma membrane-associated enzyme which utilizes NAD(P)H for the generation of ROS during macrophage activation.[34] Indeed, several investigators have reported an increase in ROS generation in phagocytic cells following He–Ne laser irradiation.[8,35] Interestingly, in their studies on the effect of He–Ne laser irradiation on spleen cells, Karu *et al*. observed a peak in the generation of ROS at a dose of ~200 J m^{-2}. This is the same as the irradiation dose found to yield the maximum NAD(P)H level observed in our study .[35] Similarly, Klebanov *et al*.[8] have also observed an increase in zymosan-stimulated ROS generation in human leukocytes at He–Ne laser irradiation doses in the range 250 to 500 J m^{-2}. It has been shown that ROS generated during immune stimulation by opsonized zymosan or latex beads contribute to changes in cell membrane fluidity in polymorphonuclear leukoctes and macrophages.[36,37] These changes in membrane fluidity may lead to regulation of macrophage functions *via* a change in the lateral mobility of various regulatory proteins present in the plasma membrane.[20,21]

To sum up, the results obtained show that He–Ne laser irradiation leads to significant modulation of a number of functional parameters of macrophages and thus leads to significant stimulation of macrophage activity. The stimulation of macrophage activity by He–Ne laser irradiation may lead to an increase in host immune competence against pathogens, contributing to the observed therapeutic effects of low level red light irradiation.

Conclusions

To conclude, the results of our studies show that He–Ne laser irradiation leads to stimulation of macrophage activity, as evidenced by a change in several of their functional parameters [lysozyme, cathepsin and phagocytic activity, membrane fluidity, and intracellular NAD(P)H level]. Although significant correlations were observed between lysozyme activity, cell size and membrane spreading on one hand, and cathepsin activity, membrane fluidity and intracellular NAD(P)H concentration on the other, the reasons for these are not yet fully understood and require further study.

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