Selective RPE-Photodestruction: Mechanism of Cell Damage by pulsed laser irradiance in the ns to µs time regime

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ABSTRACT

The subject of this study was to investigate the threshold radiant exposures for bubble formation at single porcine melanosomes in suspension and for porcine RPE cell damage when using pulse durations in the ns to µs time regime. A frequency doubled Nd:YLF laser (λ=527 nm) with adjustable pulse duration between 250 ns and 3 µs and a Q-switched Nd:YAG laser (λ=532 nm, τ=8 ns) were used for the single pulse irradiation. Fast flash photography was applied to probe vaporization around individual melanosomes while a fluorescence viability assay was used to probe cell vitality. Applying single ns laser pulses to RPE cells, an ED_{50} threshold radiant exposure of 84 mJ/cm² was determined, which is close to the vaporization threshold around single melanosomes. When irradiating with pulse durations of 3 µs, a threshold of about 223 mJ/cm² was measured, which is only 40% lower of the vaporization threshold around the single melanosome at that pulse width. This can be explained with heat contribution from adjacent melanosomes, which increases towards longer pulse durations. Calculations are in good agreement with the experimental results when assuming a surface temperature at the melanosome of 140 °C and an absorption coefficient of 8000 cm^{-1} to initiate vaporization. It can be concluded that the origin of RPE cell damage for single pulse irradiation with a duration of 8 ns results from transient microbubbles around the melanosomes, which lead to a transiently increased cell volume and subsequently a rupture of the cell structure. It is also likely that the same effect plays the major role when using pulse durations up to 3 µs.

Key words: selective photocoagulation, RPE, fast flash photography, fluorescence microscopy, melanosome, viability assay, cavitation bubble, pulse stretched laser

1. INTRODUCTION

A variety of retinal diseases such as diabetic macular edema, drusen and central serous retinopathy are thought to be associated with a declined function of retinal pigment epithelial cells. A method for selective destruction of these cells without causing adverse effects to adjacent tissue, especially to the photo receptors, seems to be an appropriate treatment[1], presuming that the destroyed cells will be replaced by proliferation or migration of neighboring intact RPE cells post treatment. The main chromophores in the fundus are melanosomes inside the RPE cells which absorb about 50% of the incident light[2]. Irradiating the fundus with a train of µs laser pulses in order to achieve high peak temperatures around the melanosomes, RPE cells were thought to be coagulated, while only a low sublethal temperature increase in the adjacent tissue structures is obtained[3]. The selective effect on RPE cells has first been demonstrated by Roider in rabbits by using 5 µs Argon-ion laser pulses at 514 nm at a repetition rate of 500 Hz[1]. ED_{50} threshold radiant exposures of 188 mJ/cm² and 32 mJ/cm² (equivalent top hat beam diameter) were found for single and 500 pulses, respectively. Fluorescein angiography was applied to visualize the ophthalmoscopically invisible effects. The selective destruction of the RPE cells sparing the photo receptors was proven by histologic examinations at different times post treatment. A first clinical trial has already proved the concept of selective RPE-destruction and demonstrated the clinical potential of this technique[4].

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Apart from a pure thermal effect, a thermo-mechanical damage of the cells has to be taken into account: Lin and Kelly heated microabsorbers in suspension with 30 ps laser pulses and demonstrated vaporization around the particles[5]. A threshold radiant exposure for microbubble formation around bovine melanosomes was found to be 55 mJ/cm^2 at a wavelength of 532 nm. Irradiating RPE cells at the same radiant exposure, intracellular cavitation effects were documented and only non-viable cells were found in a cell viability essay post irradiation[6]. They concluded that cell death is caused by thermo-mechanical disruption of the cell structure due to the significantly increased cell volume during bubble lifetime. Roider also postulated a mechanical effect to cause RPE cell damage when repetitively applying a train of 200 ns pulses to rabbit eyes[7].

Subject of this study is to investigate, whether this thermo-mechanical damage mechanism may also be responsible for selective RPE cell damage when laser pulse durations in the ns to µs time regime are used. Experiments using single pulse exposure were conducted using a Q-switched and pulse-stretched Nd:YLF laser emitting pulse durations between 250 and 3000 ns at a wavelength of 527 nm. A Q-switched, frequency doubled Nd:YAG laser at a wavelength of 532 nm served for an 8 ns exposure. Single porcine melanosomes in suspension were irradiated and bubble formation threshold was investigated by fast flash photography. Porcine RPE cell layers were irradiated and cell viability was investigated with a Calcein-AM fluorescence essay. Heat diffusion during irradiation was calculated in order to correlate the experimental findings with the temperatures induced at the melanosomes.

2. MATERIALS AND METHODS

2.1 Laser

An arc-lamp excited, intracavity frequency doubled Nd:YLF laser (Quantronix Inc., model 527DP-H) was modified with an active feedback electro-optical Q-switched system to generate pulse durations up to several µs at a wavelength of 527 nm. Operating the system in the normal Q-switched mode, the laser emits pulses of typically 250 ns in duration with pulse energies of several millijoules at a repetition rate of 500 Hz. In order to extend the pulse duration, a transient high voltage course was applied to the Pockel's-cell to increase cavity losses during pulse emissions. If the losses of the cavity are equal to the gain of the laser medium at any time during pulse emissions, a pulse of constant power is emitted until a depletion of the inversion as calculated in Fig, 1a. A high voltage course needed for this purpose is shown in Fig. 1b, a more detailed description of the pulse-stretched process is found elsewhere[8]. The energy was transmitted by a 100 µm core diameter fiber (Ceram Optec GmbH, Optran UV-A 105/125/250, NA=0.22), the length of the fiber was 200 m in order to minimize spatial and temporal intensity modulation due to speckle formation at the distal fiber tip. Pulse durations of 8 ns were generated with a flashlamp pumped Q-switched, frequency doubled Nd:YAG laser (Spectron Inc.) at a wavelength of 532 nm.

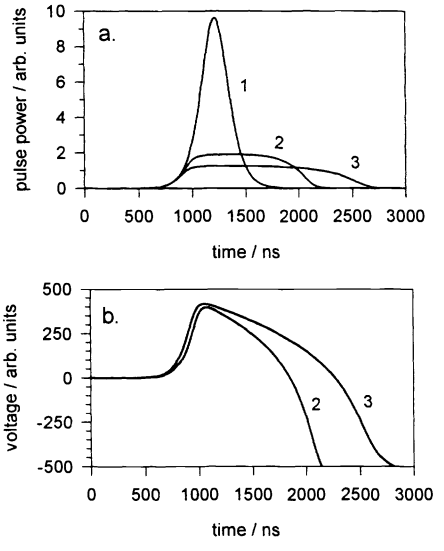

Fig. 1 a: Calculations of a Q-switched Nd:YLF laser pulse (1) and two stretched pulses (2,3) achieved by applying a correspon ding high voltage course as shown in b

2.2 Porcine eye model, viability essay and experimental setup

RPE cell samples and melanosomes were harvested from freshly enucleated porcine eyes. Melanosomes were extracted from RPE cells and suspended in distilled water. Several drops were placed on standard microscope slides, covered with cover slips, which were sealed to prevent evaporation of water. In order to probe RPE cell vitality, Calcein-AM (Molecular Probes Inc.) was used as a fluorescent dye marker to tag living cells. Calcein-AM is transformed in living cells to Calcein by esterases. Calcein can be excited with blue light (excitation maximum at 490 nm) to fluoresce in the green spectral region. Calcein-AM was buffered in a phosphate solution in a concentration of 2µg/ml and 20 ml were dropped onto the RPE cell layer, either prior or post irradiation.

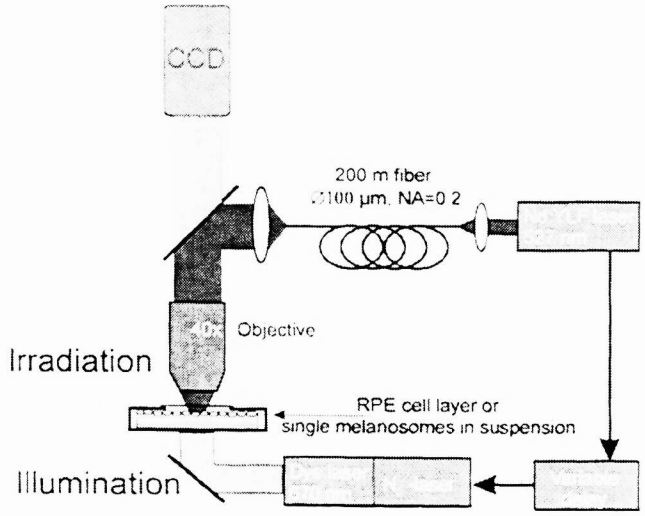

Fig. 2 Experimental set-up for irradiation of single melanosomes in suspension or a complete RPE-cell layer including fast flash photography to observe bubble formation around melanosomes

The experimental set-up to irradiate the samples, either melanosomes in suspension or complete RPE cell layers, is shown in Fig. 2. The distal fiber tip was imaged onto the object plane to a diameter of 47 µm, an almost top hat spatial beam profile with spatial intensity modulation below 10% was obtained. A suspension of melanosomes was collinearly illuminated with a N_2-pumped dye laser (670 nm, 1 ns) to probe vaporization occurring around the melanosomes during irradiation. A variable electronic delay was used to trigger the N_2 laser with the Nd:YLF or Nd:YAG laser, allowing to shift the probe pulse relative to the irradiation laser pulse.

2.3 Threshold irradiance

The vaporization threshold radiant exposure on single melanosomes was determined by reducing the pulse energy with a variable attenuator until bubble formation was only found in 50% of the cases.

RPE cell samples were prepared by taking circular section of 1 cm^2 of the eye fundus including the sclera and carefully pealing off the neural retina. Marker lesions using pulse energies far above damage threshold were placed on the sections. Directly after irradiation, Calcein-AM was added to the probe and non-fluorescing cells were counted under a fluorescence microscope within 30 min post irradiation. Each spot covered about 10 RPE cells, and from the ratio of vital to death cells, the ED_{50} was evaluated.

2.4 Temperature calculations

Temperatures were calculated with an analytical solutions of the heat diffusion equation of spherical particles[9]. The melanosomes were assumed as spherical particles of 1 µm in diameter with the thermal properties of the surrounding water, which were homogeneously heated with a laser pulse of constant power. The transient temperature course inside and around the melanosomes was calculated as a function of radiant exposure, pulse duration and absorption coefficient.

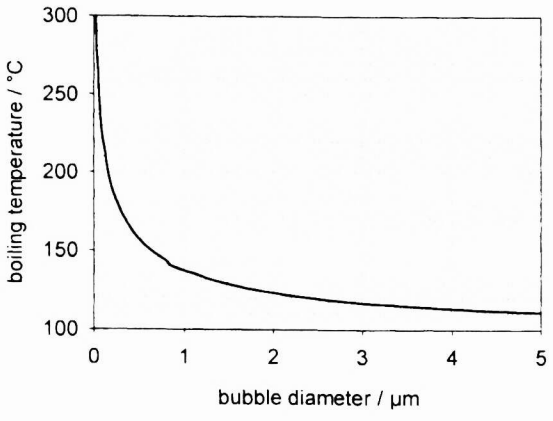

Fig. 3 Required temperature to start bubble formation in water as a function of the initial bubble diameter

Assuming a vapor bubble occurs after a certain temperature at the surface of the melanosome is reached, the vapor pressure must overcome the hydrostatic and the surface tension pressure for bubble growth. The pressure inside the gas bubble due to surface tension of water significantly increases with decreasing bubble radius r according to $P=2*\sigma/r$ [10]. Due to the high pressure for small bubbles, the boiling temperature around the melanosome is elevated. Fig. 3 depicts the bubble radius as a function of the boiling temperature of water for $\sigma_{100°C}$ = 58.8 mN/m, neglecting the small temperature dependence of σ[10]. The initial bubble formation requires to overcome a pressure of 3.35 bar for a bubble diameter of 1 µm and thus at least a temperature of 137°C is needed at the melanosome-water interface. For the calculations, we assumed that vaporization occurs when the surface temperature of a melanosome has reached 140°C. As the only remaining parameter, the absorption coefficient of the melanosome was adjusted to fit the experimental data.

3. RESULTS

3.1 Pulse stretched Nd:YLF laser

Typical pulse shapes achieved with the Nd:YLF laser system under constant pump conditions at a pulse repetition rate of 500 Hz is shown in Fig. 4. Under normal Q-switched conditions, a pulse energy of typically 1 mJ is achieved at a pulse duration of 250 ns full width at half maximum (FWHM). Extending the laser pulses unavoidable leads to a reduced pulse energy since increased cavity losses lead to an increased final population inversion density of the laser medium[8,11]. Typically at a pulse duration of 1 µs, 55% of the unstretched Q-switch energy is extracted, which is further reduced to about 35% at a pulse duration of 3 µs.

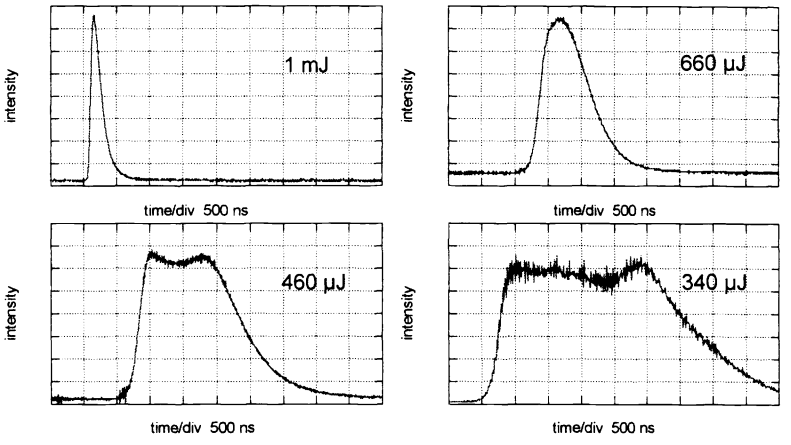

Fig. 4 Typical pulse shapes between 250 ns and 3µs FWHM at a wavelength of 527 nm with corresponding pulse energies achieved with the same lamp pump current

3.2 Bubble formation thresholds at single melanosomes and ED_{50} damage threshold of RPE cells

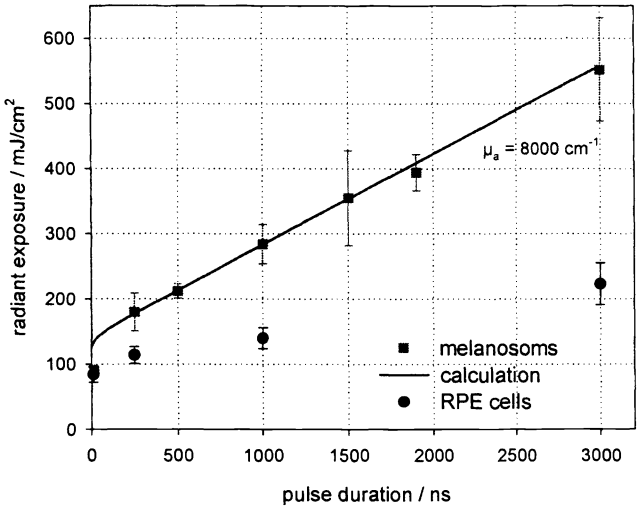

Fig. 5 Threshold radiant exposures for bubble formation on single melanosomes in suspension and ED_{50} damage thresholds of RPE-cells. The solid line represents a calculation of the radiant exposure assuming bubble formation starts at a melanosome surface temperature of 140°C, using an absorption coefficient of 8000 cm^{-1}.

Fig. 5 shows the radiant exposures at bubble formation threshold of single melanosomes and the ED_{50} thresholds for RPE cell damage when irradiated with one single laser pulse. Applying 8 ns pulses, the threshold radiant exposure for melanosomes and RPE cells is nearly the same with 84 and 90 mJ/cm^2, respectively. For bubble formation at single melanosomes, the threshold increases by nearly a factor of two at pulse durations of 250 ns and further increases almost linearly with pulse duration up to 552 mJ/cm^2 at a pulse duration of 3 µs. RPE cell damage radiant exposure also increases with pulse duration, however, significantly slower as observed on melanosomes. At a pulse duration of 3 µs, only 40% of the energy is needed for 50% cell damage.

3.3 Calculation of heat diffusion at the melanosomes

The calculated threshold radiant exposures are in good accordance with the experimental data when an absorption coefficient of μ_a=8000 cm^{-1} is assumed. The solid line through the data points in Fig. 5 represents the calculated radiant exposures to reach a melanosome surface temperature of 140°C at the end of the laser pulses, respectively. Fig. 6 further demonstrates the temperature course inside and

around a single melanosome of 1 µm in diameter. When applying a laser pulse width below 10 ns, conditions for thermal confinement are fulfilled. The temperature increases linear with time and a radiant exposure of 129 mJ/cm² is needed to induce a surface temperature of 140°C at the melanosome-water interface. If the pulse duration is extended to 3 µs, a 4.3 times higher radiant exposure is needed to induce the same temperature. During the µs exposure, the temperature inside the melanosome increases much slower than linear towards saturation at the equilibrium temperature.

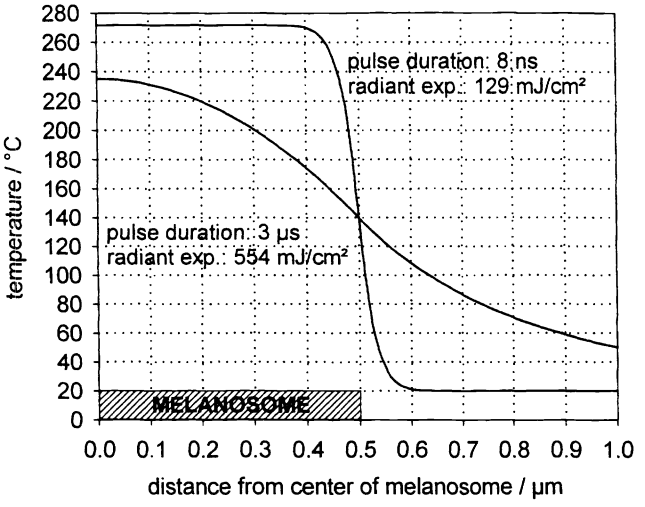

Fig. 6 Calculations of the radiant exposures needed to achieve a temperature of 140°C at the surface of the melanosome at the end of an 8 ns and 3 µs laser pulse, respectively.

4. DISCUSSION

In this study, the threshold radiant exposures to cause microbubble formation around single porcine melanosomes in suspension and the ED_{50} damage radiant exposure of RPE cells have been investigated for single pulse irradiation from the ns to the µs time regime.

With respect to the irradiation of single melanosomes, we observed bubble formation at a threshold radiant exposure of 84 mJ/cm² for pulse durations of 8 ns. In a similar experiment using bovine melanosomes, Lin and Kelly found a threshold radiant exposure of 55 mJ/cm² for 20 ns and 30 ps laser pulses at a wavelength of 532 nm[5,12]. When comparing these thresholds, one has to consider the size of the different melanosomes, which are of cylindrical shape with the long axis in the order of 1 µm for porcine and 2.5 µm for bovine melanosomes. Assuming similar optical and thermal properties, a lower threshold radiant exposure is expected for the larger bovine melanosomes, according to the reduced required boiling temperature at the surface as shown in Fig. 3. Thus, the results of both studies are in a quite good agreement.

Using the Nd:YLF laser, we found an almost linear increasing threshold radiant exposure with pulse durations from 250 ns to 3 µs. However, it is obvious that the threshold for 8 ns is lower than expected from the calculations. Despite the different laser systems used, the assumption of homogeneously heating the melanosome is not fulfilled when using short exposure times. Due to the strong absorption, the surface temperature of the melanosomes on the illuminated front side of the particles should be higher than on the back side. This effect tends to lower the threshold radiant exposure for pulse durations shorter than the time of thermal confinement.

In order to analyze the experimental findings, we performed temperature calculations inside and in the surroundings of the irradiated melanosomes. Thermal models and calculations of heat diffusion around microabsorbers have already been published by different groups: Temperatures were calculated around melanosomes, in the RPE and neural retina in order to describe selective RPE photocoagulation[3,13]. Gerstman made temperature assumptions to calculate bubble radii as a function of radiant exposure in order to analyze the experimental findings related to minimal visible lesions after ns laser exposure[14]. Besides the thermal properties of the microabsorbers, the most important parameter to calculate reasonable temperatures is the absorption coefficient. Most groups working with a granule RPE-model use an absorption coefficient between 1000 – 2500 cm^{-1} in the green spectral range[14-17]. However, recent experiments seems to indicate that the absorption coefficient of a single melanosome is significantly higher. Strauss[18] concluded from shock wave modulation

around melanosomes that the absorption coefficient should be 6000-8000 cm^{-1}, experiments done by Kelly[12] lead to a bovine absorption coefficient of around 9900 cm^{-1} for ns pulse durations.

Due to the lack of accurate data, we used the absorption coefficient as a fit parameter to model threshold radiant exposure versus pulse duration. Going this way, an assumption about the threshold temperature for bubble formation has to be made. We assumed that vaporization always occur at a certain surface temperature of the melanosome. We chose 140°C as the boiling temperature required to create bubbles of 1 μm in diameter as shown in Fig. 3. An experimental confirmation for an increase of the boiling temperature was given by Kelly, who performed threshold measurements on bovine melanosomes with ns pulse durations at different water temperatures. Extrapolating the data to zero threshold radiant exposure leads to a temperature of around 150°C[12]. Furthermore, the thermal parameters of water were used in the calculations for the melanosomes due to the lack of more accurate data. Although the heat capacity c and density ρ of melanin was reported to be c=2.5 J/(g*K) and ρ=1.4 g/cm^3, however, in the heat diffusion equation only the product of c*ρ appears, which is just 15 % lower than of water[19].

Using the thermal properties of water and the assumption that 140°C is the required surface temperature of melanosomes to start vaporization, an absorption coefficient of μ_a = 8000 cm^{-1} best fits to the data as shown in Fig. 5. This is in accordance to the recently published high values for the melanosome absorption coefficients. However, other parameter sets can also be used to produce reasonable fits, since ΔT is proportional to μ_a and a smaller product of c*ρ to better match melanin properties can be compensated for a lower μ_a.

The origin of RPE cell damage as a consequence of short-pulsed laser irradiation may either be thermal damage of the cell as it takes place for cw-irradiation, or be thermo-mechanical due to a rupture of the cell structure: Melanosomes occupy a significant amount of the RPE cell volume. Since bubble formation occurs simultaneously around all melanosomes, the cell volume could increase transiently and may lead to mechanically disrupted cell membrane. Kelly demonstrated bubble formation inside bovine RPE cells and showed that the threshold radiant exposures for bubble formation and cell death are the same for ps-laser light exposure[6].

With regard to the RPE-damage radiant exposure it is obvious that vaporization threshold and cell damage are nearly the same for ns-exposure. However, the cell damage threshold is significantly lower at pulse durations in the μs time regime. Assuming bubble formation as the primary mechanism to induce cell damage, the difference can be explained by the heat contribution of surrounding melanosomes, which contribute to the surface temperature of a given melanosome, since the condition of thermal confinement is no longer fulfilled for μs pulse durations. This effect reduces the threshold radiant exposure compared to a single melanosome. Fig. 6 demonstrates this fact: Assuming a second melanosome at a distance of 0.5 μm, heat flow from the first one increases the surface temperature by about 30°C. The heat contribution of a field of melanosomes to the surface temperature at a single melanosome increases with longer pulse durations.

As shown in transmission electron microscopy (TEM), the distance between the individual melanosomes in porcine RPE cells varies between almost zero and a few μm[20]. More accurate calculations taking into account a field of melanosomes demonstrate that a 60% decrease in threshold radiant exposure can be calculated, when assuming μs laser pulse durations and spacing of the melanosomes in the order of 1.5 μm (center-center). Thus, thermo-mechanical cell damage is most likely responsible for single pulse exposure up to pulse durations of 3 μs.

5. CONCLUSION

It was shown that the vaporization threshold radiant exposure around single melanosomes increase linearly with pulse duration from 250 ns up to 3 μs regime, which can be explained by heat diffusion. Assuming a certain, constant surface temperature of the melanosome to be required to start bubble formation, calculations of the threshold radiant exposure is in good agreement with experimental findings when using a melanosome absorption coefficient as high as 8000 cm^{-1}. Irradiating complete RPE cells with μs laser pulses, heat contribution from surrounding melanosomes has to be taken into account when regarding vaporization at a single melanosome. It is most likely that the origin of RPE cell damage for single pulse irradiation up to pulse durations in the μs-time regime results from vaporization around the melanosomes and subsequent disruption of the cell structure.

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