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A Novel Gene Delivery System for Mammalian Cells

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Abstract. *Although gene therapy holds great promise for the treatment of both acquired and genetic diseases, its development has been limited by practical considerations. Non-viral efficacy of delivery remains quite poor. We are investigating the feasibility of a novel lipid-based delivery system, cochleates, to deliver transgenes to mammalian cells. Rhodamine-labelled empty cochleates were incubated with two cell-lines (4T1 adenocarcinoma and H36.12 macrophage hybridoma) and primary macrophages in vitro and in vivo. Cochleates containing green fluorescent protein(GFP) expression plasmid were incubated with 4T1 adenocarcinoma cells. Cellular uptake of labelled cochleates or transgene GFP expression were visualised with fluorescence microscopy. 4T1 and H36.12 lines showed 39% and 23.1% uptake of rhodamine-cochleates, respectively. Human monocyte-derived macrophages and mouse peritoneal macrophages had 48±5.38% and 51.46±15.6% uptake of rhodamine-cochleates in vitro. In vivo 25.69±0.127% of peritoneal macrophages were rhodamine-positive after intra-peritoneal injection of rhodamine-cochleates. 19.49±10.12% of 4T1 cells expressed GFP. Cochleates may therefore be an effective, non-toxic and non-immunogenic method to introduce transgenes in vitro and in vivo.*

Gene therapy holds huge promise for the treatment of a range of diseases from cancer to genetic disease (mutations or inherited genetic disorders). However, at the present time, the use of gene therapy is limited by the lack of suitable methods for introducing the gene into cells.

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The use of viral vectors, while providing a useful research tool, is limited by handling difficulties (Class 2 organisms), toxicity and immunological considerations. For example, the immunogenic nature of adenovirus (AdV) means that their use is limited to single dose, which is unlikely to be sufficient for many gene therapy applications although AdV vectors have been used to deliver single dose angiogenic gene therapy for diabetes-induced impairment of neovascularisation in mice (1). In addition to immunogenicity concerns, there is also the possibility of these vectors being oncogenic, in particular retroviral vectors which are incorporated into the host DNA (2).

Although a range of delivery options have been investigated including liposome systems, naked DNA, electroporation, ballistic guns, microinjection of DNA, receptor-mediated gene transfer and the use of plasmid or viral vectors, they are all limited by practical considerations such as toxicity or inefficient delivery (2-6). Delivery of naked DNA is highly inefficient and many liposome systems are also limited by relative inefficency (7).

Cochleates (Biodelivery Sciences International) are highly stable lipid bilayer structures which are non-toxic and non-inflammatory. The molecules housed within the lipid bilayers, and the cochleates themselves, are protected from degradation by this unique tightly-packed, multilayer structure. Cochleates are stable phospholipid-divalent cation precipitates composed of phosphatidyl serine (PS) and calcium (8-10). In brief, the material to be formulated (in our case, empty cochleates with rhodamine label covalently attached to the headgroup of phospholipid, or a green fluorescent protein (GFP) expression vector) is added to a suspension of liposomes comprised mainly of negatively-charged lipids. Divalent cations, usually calcium, are added, which induce the collapse and fusion of liposomes into large sheets of lipid bilayers that then spontaneously roll up to form cochleates. Cochleates are stacked sheets or a

continuous lipid bilayer sheet rolled into a spiral, resulting in a multilayer structure with no internal aqueous space. As the interior of cochleates is essentially free of water and resistant to oxygen penetration, molecules housed within cochleates are protected from degradation and encochleated biological molecules are highly stable. This unique structure protects components within the interior of the cochleate even if the exterior layers of the cochleate are exposed to harsh conditions. Thus encochleated materials are resistant to chemical or enzymatic attack and, as the cochleates are stable at low pH, cochleate formulations can survive the gastrointestinal tract or other mucosal surfaces, making them ideal for oral or mucosal delivery of biological molecules (11).

Many naturally occurring membrane fusion events involve the interaction of calcium with negatively-charged phospholipids such as phosphatidylserine (12-15). When cochleates come in proximity to a cell membrane, calcium-induced perturbation and re-ordering of the cell membrane results in a fusion event between the outer layer of the cochleate and the cell membrane and delivery of the encochleated material (in our case, GFP plasmid) into the cytoplasm of the target cell. Cochleates may also be taken up by endocytosis and fuse from within endocytic vesicles delivering the DNA directly into the cell (10,12).

The aim of our study was to investigate the feasibility of using cochleates to deliver a functional transgene. We investigated cellular uptake of rhodamine-labelled cochleates (rho-cochleates) in cell lines and primary cells, *in vitro* and *in vivo* and the expression of GFP delivered to tumour cells using cochleates.

Materials and Methods

Cells and reagents. The 4T1 mammary adenocarcinoma cell line was a gift from Dr Fred Miller, Duke University, USA. The H36.12 macrophage hybrid cell line was purchased from ATCC. Cells were maintained in DMEM (4000 mg/L glucose) or RPMI1640 with 50 U/ml penicillin, 50 μg/ml streptomycin (P/S) and 10% foetal calf serum in a 5% CO_2-95% air atmosphere at 37˚C unless otherwise stated. All cochleate formulations were kindly provided by Biodelivery Sciences International. Rhodamine-labelled cochleates (rho-cochleates) were provided at a concentration of 10 mg/ml. Cochleates containing the green fluorescent protein (GFP) expression plasmid were provided at concentration of 3 mg/ml. Weight ratio of soy PS lipid to plasmid was 10:1. Cochleates were stored at 4˚C and protected from light. All culture media and Dulbeccos phosphate-buffered saline, without calcium and magnesium, was purchased from GIBCO-BRL. Thioglycollate broth was purchased from Sigma-Aldrich. Fluorescein isothiocyanate isomer1 (FITC) conjugated rat anti-mouse macrosialin antibody was purchased from Serotec.

Animals. Female 10- to 12-week-old BALB/c mice (Charles River Institute, Margate, Kent, UK) were used. The animals were acclimatised for 1 week and caged in groups of five or less in an air-conditioned room at ambient temperature of 21-22˚C and 50% humidity under a 12-h light-dark cycle (lights at 08.00). Animals were housed in a licensed biomedical facility (RCSI Department of Surgery, Beaumont Hospital, Ireland) and all procedures were reviewed by RCSI Ethics Committee, carried out under animal license guidelines of the Department of Health, Ireland and in accordance with the UK Co-ordinating Committee on Cancer Research (UKCCCR) Guidelines for the Welfare of Animals in Experimental Neoplasia (16). Animals had *ad libitum* access to animal chow (WM Connolly & Sons Ltd, Kilkenny, Ireland) and water.

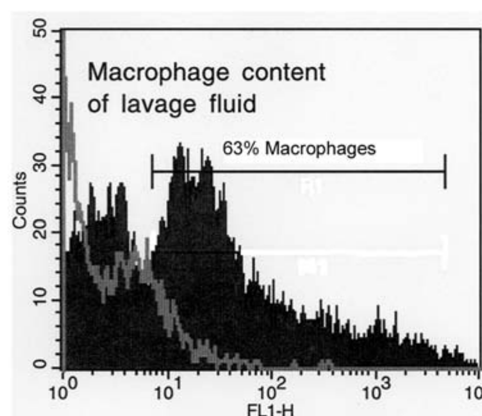

Figure 1. *Representative flow cytometry analysis diagram of peritoneal lavage fluid. Peritoneal lavage fluid was incubated with 10 μg/ml FITC-labelled rat anti-mouse macrosialin antibody for 30 minutes and the percentage macrophages determined by flow cytometry.*

In vitro evaluation of rho-cochleate uptake by 4T1 and H36.12 cell lines. Cells were seeded at $1x10^5$ cells/well in a 4-well chamber slide and allowed to adhere overnight. Cochleates were vortexed immediately before use, diluted in either RPMI1640 or DMEM with 1 mM $CaCl_2$ to 20 μg/ml. Diluted cochleates were added to equal volume of media on cells, resulting in final cochleate concentrations of 10 μg/ml. Cells were incubated for 1 or 6 hours before being viewed under fluorescent microscope using Rhodamine filter (Excitation 543/68 nm, Emission 585/625 nm) to determine levels of rhodamine-cochleate uptake.

In vitro uptake of rho-cochleates by human monocyte derived macrophages. Forty mls of human blood (n=3) was centrifuged on a density gradient of histopaque 1119 and 1077(Sigma). Monocyte/lymphocyte layer was recovered and resuspended in DMEM containing P/S and 20% autologous plasma. Monocytes were matured and activated with human-interferon gamma (hIFN-γ) as described previously (17). Cochleates were diluted in DMEM containing P/S and 20% autologous plasma and added to cells as described above. Cells were incubated for 1 or 6 hours. Cells were viewed using a fluorescent microscope as above.

In vitro evaluation of cochleate uptake by primary macrophages. Ten-week-old BALB/c mice (n=3) received a 1.5 ml intra-peritoneal injection of 3% thioglycollate broth. Four days post injection, the peritoneal cavity was lavaged with 5 mls cold saline. Flow cytometry

analysis of lavage fluid using fluorescein isothiocyanate isomer1 (FITC)-labelled rat anti-mouse macrosialin antibody at 10 μg/ml for 30 minutes (Serotec) showed that the lavage fluid contained approximately 63% macrophages (Figure 1). Cells were seeded into 4-well chamber slide at 200,000 cells per well. Macrophages were allowed to adhere for 3 hours and non-adherent cells were removed by washing twice with PBS. Fresh RPMI1640 containing 10% FCS and 1mM $CaCl_2$ was added to the cells and cochleates diluted in RPMI1640 and 1mM $CaCl_2$ were then added dropwise to cells to a final cochleate concentration of 10 μg/ml. Cells were incubated with rho-cochleates for 3 hours and then viewed as above using fluorescent microscope.

In vivo uptake of rhodamine-labelled cochleates by macrophages. Following thioglycollate elicitation to the peritoneal cavity of BALB/c mice (n=3) as above, 500 μg of rho-cochleates were injected into the peritoneal cavity. Six hours or 24 hours later, peritoneal macrophages were recovered and seeded at 200,000 per well of 4-well chamber slides. Cells were allowed adhere for 3 hours, non-adherent cells were removed by washing twice with PBS. Rho-cochleate uptake was visualised using a fluorescent microscope as above.

In vitro delivery of encochleated green fluorescent protein (GFP) expression vector. 4T1 cells were seeded at 5,000 cells per well in 4-well chamber slide (n=4) in RPMI 10%FCS, P/S and 4mM $CaCl_2$ and allowed to adhere overnight. Cochleates containing the pcDNA5/TO GFP expression plasmid (Invitrogen) were diluted in RPMI1640 containing 4mM $CaCl_2$, with final concentrations of 300, 150, 75, 37.5, 18.75, 9.375 and 4.735 μg/ml. Diluted cochleates were added dropwise to cells and viewed under fluorescent microscope with GFP filter (Excitation 470/40 nm, Emission 525/50 nm) daily for seven days.

Results

In vitro uptake of rhodamine-labelled cochleates by cell-lines. 23.1% of H36.12 cells were rhodamine positive after 6 hours incubation with 10μg/ml rhodamine-cochleates. The 4T1 cell line appeared to take up the rho-cochleates more readily with 39% of cells positive after 6 hours (Figure 2). At 1 hour rhodamine cochleates were seen fusing to the cell membranes with the peak uptake seen at 6 hours. Rhodamine signal was still visible at 24 hours, but at lower levels (19.3% 4T1 and 14.1% H36.12) indicating that the rhodamine label and perhaps the cochleates are being degraded.

In vitro uptake of rhodamine-labelled cochleates by primary cells. 7.24%±3.77 of monocyte-derived macrophages were rhodamine positive 1 hour after the addition of rho-cochleates. After five hours 33.3% of these monocyte-derived macrophages were positive for rhodamine. After 24 hours incubation with rhodamine-cochleates 48±5.48% of human primary monocyte-derived macrophages were rhodamine-positive (Figure 3). Mouse macrophages were elicited to peritoneal cavity by thioglycollate injection and recovered by lavage. After 1 hour *in vitro* incubation with rho-cochleates, 51.46±15.6% of murine peritoneal macrophages were rhodamine-positive (Figure 4). After 3 hours, 38.07±14.95% were rhodamine-positive.

In vivo uptake of rhodamine-labelled cochleates by primary murine macrophages. Mouse macrophages were elicited to the peritoneal cavity by thioglycollate injection. Four days later 500 μg rho-cochleates were injected intraperitoneally. Macrophages were recovered by lavage. Six hours after intra-peritoneal injection of rho-cochleates, 25.69±0.1273% of recovered macrophages were rhodamine-positive (Figure 5). Twenty-four hours post *i.p.* injection of cochleates, 18.55±2.5% of recovered macrophages were rhodamine-positive.

In vitro delivery of green fluorescent protein reporter gene to 4T1 cell line. 4T1 cells were incubated *in vitro* with cochleates containing GFP-expression plasmid for up to 7 days and viewed daily to monitor GFP expression. From 48 hours GFP expression was observed. 14.44±8.5% of 4T1 cells were GFP-positive at this stage. This GFP expression was sustained for up to 7 days, with maximum transfection levels of 19.49±10.12% seen 96 hours after addition of 37.5 μg/ml cochleates to cells (Figure 6). Lower levels of GFP expression were seen at the other concentrations examined. (Data not shown)

Discussion

In this study we have shown that cochleates are taken up by both cell lines and primary cells *in vitro* and *in vivo*. Primary cells, macrophages in particular, are notoriously difficult to transfect with non-viral methods (18). The high levels of cell labelling with rhodamine, after incubation with rho-cochleates, demonstrate important prerequisite steps towards the goal of efficient transfection, namely access to the target cell population and crossing the plasma membrane (potentially by fusion). The *in vitro* and *in vivo* labelling work demonstrated that cochleates are efficiently accumulated by macrophages.

The high levels of rhodamine labelling with the 4T1 and H36.12 cell lines proved the efficient ability of cochleates to target a cell population. Rhodamine degradation and diminishment of signal, probably due to intercellular enzymes, suggests that following the fusion event, cochleates gain intercellular access.

The expression of green fluorescent protein in the 4T1 cell line, peaking at 19.49%, is a higher transfection rate then we have previously seen working with these cells using a range of commercially available liposome transfection reagents. In contrast to the cationic lipid transfection reagents, this high transfection is associated with little or no cytotoxicity.

Cochleates have previously been used to improve and enhance the delivery and therapeutic effect of a clinically

Figure 2. *Rhodamine-labelled cochleate uptake by cell lines in vitro. (A) Bright-field and fluorescent images of H36.12 cells following 6-hour incubation with 10 μg/ml rhodamine-cochleates. (B) Bright-field and fluorescent images of 4T1 cells following 6-hour incubation with 10 μg/ml rhodamine-cochleates. Original magnification X200.*

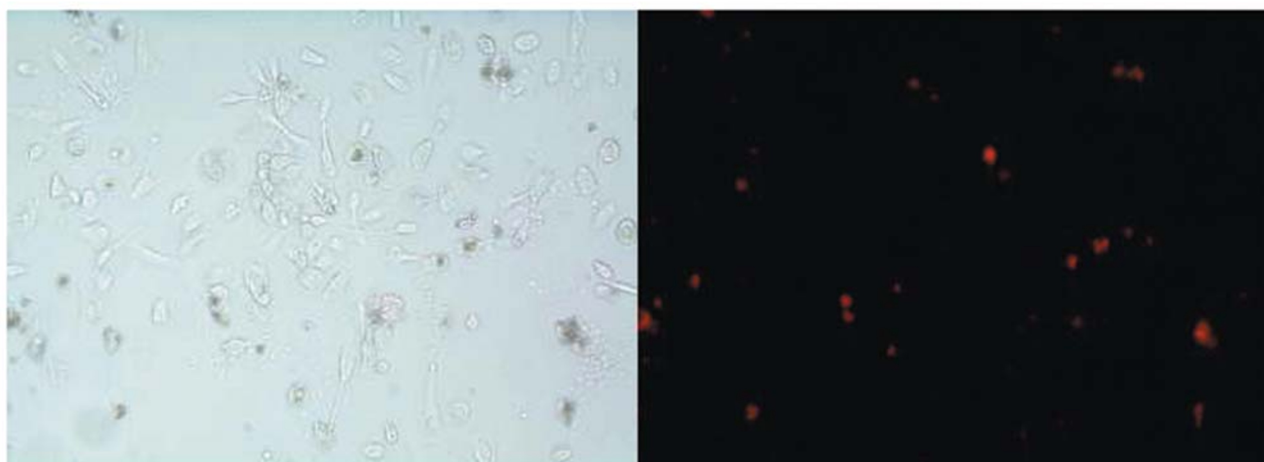

Figure 3. *Rhodamine-labelled cochleate uptake by human primary monocyte-derived macrophages in vitro. Bright-field and fluorescent field images of human monocyte-derived macrophages incubated with 10 μg/ml rhodamine-labelled cochleates for 24 hours. Original magnification X200.*

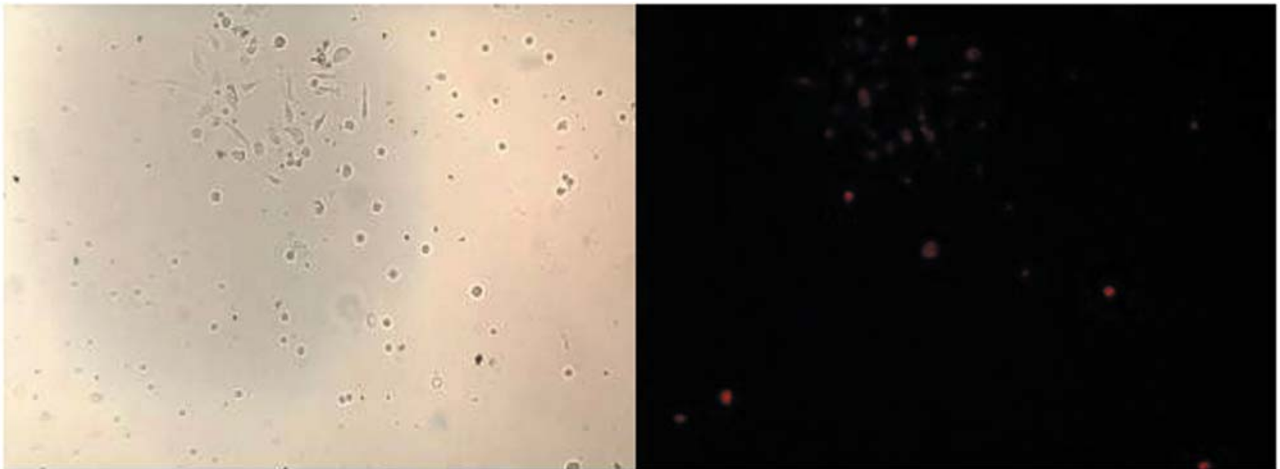

Figure 4. *Rhodamine-labelled cochleate uptake by murine primary macrophages in vitro. Bright field and fluorescent field images of mouse macrophages incubated with 10 µg/ml rhodamine-labelled cochleates for 1 hour. Original magnification X200.*

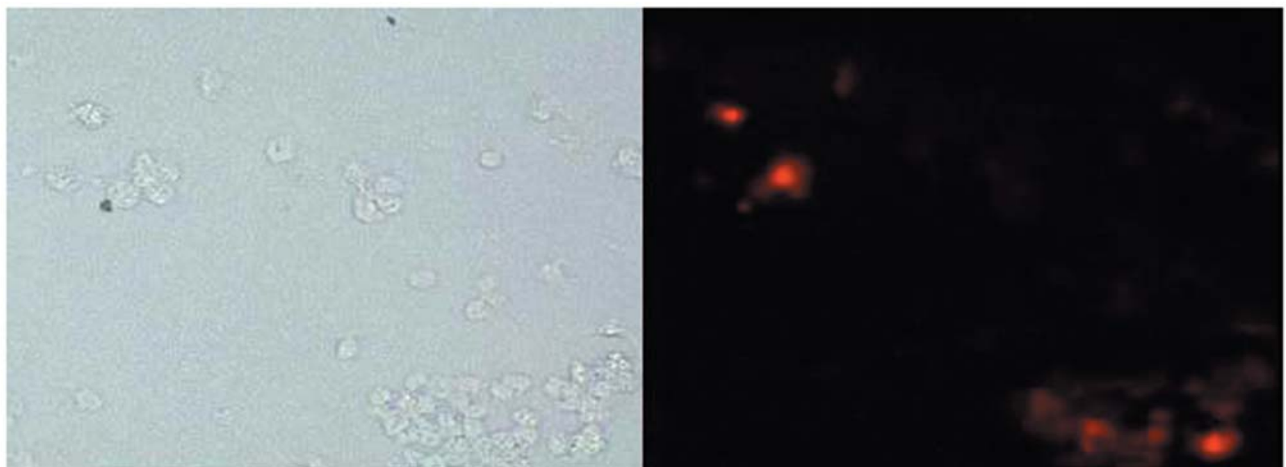

Figure 5. *Rhodamine-labelled cochleate uptake by mouse macrophages in vivo. Bright field and fluorescent field images of mouse macrophages isolated 6 hours after intra-peritoneal injection of 500 µg rhodamine-labelled cochleates. Original magnification X200.*

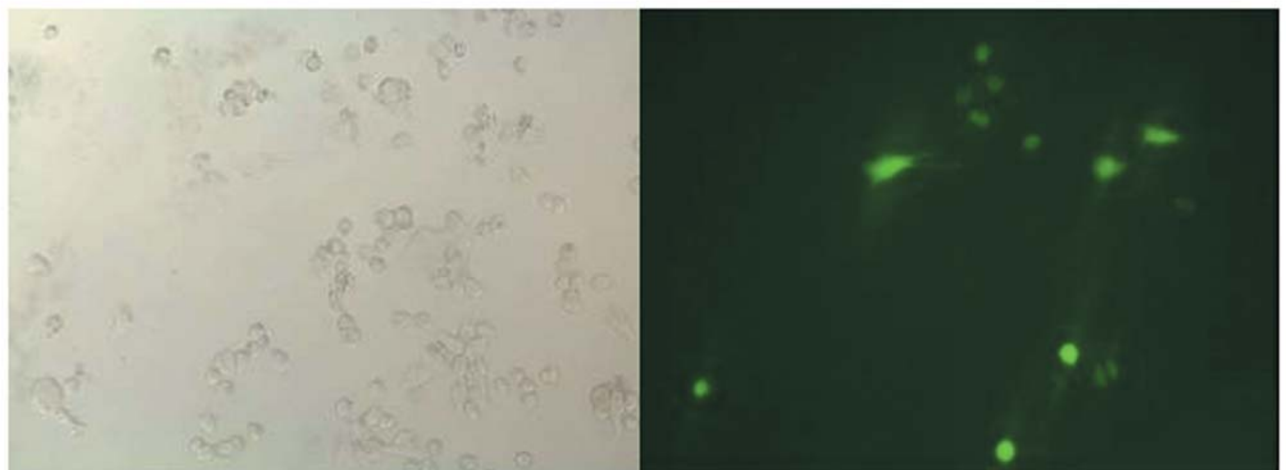

Figure 6. *Transfection of 4T1 adenocarcinoma cells with GFP plasmid-cochleates in vitro. Bright field and fluorescent field images of 4T1 adenocarcinoma cells incubated with 37.5 µg/ml GFP plasmid-cochleates for 96 hours. Original magnication X200.*

important drug, amphotericin B, known to be very effective against systemic candidiasis. In this case cochleate delivery (systemic or oral) of amphotericin B resulted in decreased toxicity, with no loss of efficacy, compared to that seen with systemic delivery (8,19). Cochleates containing viral coat proteins, or plasmid DNA encoding viral proteins, have been used as oral vaccines in mice (20). Interleukin 10 antisense oligonucleotides have also been delivered intra-peritoneally in encochleated form, inhibiting the growth of a malignant B cell line *in vivo* (21).

Because of the non-immunogenic nature of cochleates, as opposed to viral vectors, repeated doses of cochleates are possible to increase *in vivo* transfection efficiency. Repeated intra-peritoneal doses of cochleates to mice have shown that cochleates are well tolerated with no toxic, inflammatory or immune responses recorded (12).

In conclusion, our preliminary results demonstrate the feasibility of using cochleates as an alternative to viral vectors. The *in vitro* transfection results show cochleates can be used to deliver a functional transgene to mammalian cells with sustained expression (2-7 days). Our *in vivo* studies suggest that cochleates may be an effective method of *in vivo* transgene delivery. Further studies are required to optimise their ability to transfect a range of primary cells and tumours in the *in vivo* setting.

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