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ORIGINAL ARTICLE

Activation of tumor-specific T lymphocytes after laser-induced thermotherapy in patients with colorectal liver metastases

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Abstract
Purpose To asses if laser-induced thermotherapy (LITT) induces a specific cytotoxic T cell response in patients treated with LITT for colorectal cancer liver metastases.
Methods Eleven patients with liver metastases of colorectal cancer underwent LITT. Blood was sampled before and after LITT. Peripheral T cell activation was assessed by an interferon gamma (IFNg) secretion assay and flow cytometry. Test antigens were autologous liver and tumor lysate obtained from each patient by biopsy. T cells were stained for CD3/CD4/CD8 and IFNg to detect activated T cells. The ratio of IFNg positive to IFNg negative T cells was determined as the stimulation index (SI). To assess cytolytic activity, T cells were co-incubated with human colorectal cancer cells (CaCo) and cytosolic adenylate kinase release was measured by a luciferase assay.
Results IFNg secretion assay: before LITT SI was 12.73 (±4.83) for CD3+, 4.36 (±3.32) for CD4+ and 3.64 (±1.77) for CD8+ T cells against autologous tumor tissue. Four weeks after LITT SI had increased to 92.09 (±12.04) for CD3+ ($P < 0.001$), 42.92 (±16.68) for CD4+ ($P < 0.001$) and 47.54 (±15.68) for CD8+ T cells ($P < 0.001$) against autologous tumor tissue. No increased SI was observed with normal liver tissue at any time point. Cytotoxicity assay: before LITT activity against the respective cancer cells was low, with RLU = 1,493 (±1,954.68), whereas after LITT cytolytic activity had increased to RLU = 7,260 [±3,929.76 ($P < 0.001$)].
Conclusion Patients with liver metastases of colorectal cancer show a tumor-specific cytotoxic T cell stimulation and a significantly increased cytolytic activity of CD3+, CD4+ and CD8+ T cells after LITT against an allogenic tumor (CaCo cell line).

Keywords Laser-induced thermotherapy · Colorectal cancer · Liver metastases · LITT · T lymphocytes

Introduction

Colorectal carcinoma is one of the most common malignant neoplasms in the industrialized countries. For CRC with hepatic metastasis, survival is determined by the number and extent of metastases.

Surgical resection is still the gold standard for the treatment of liver metastases of colorectal cancer. However, more than 75% of these patients are unresectable for various reasons, such as size and location of the tumor [1]. Laser-induced thermotherapy (LITT) is one of the treatment options for unresectable liver tumors. Thus, it has been shown that LITT and radiofrequency thermal ablation (RFA) can achieve survival times comparable to surgery in selected patients [2, 3]. In addition it has been shown that RFA induces a significantly increased tumor-specific T cell activation in an animal model as well as in patients [4, 5].

There has been evidence from transplanted tumor models in rats, that also LITT has an immunogenic effect [6, 7].

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To our knowledge the possibility of increased tumor-specific T cell activation in patients has not been examined to date.

The aim of the present study was to determine whether an antitumor T cell response can be detected in patients treated with LITT. Therefore, we studied the (CD3+, CD8+ and IFNg+) T cell and T helper cell (CD3+, CD4+ and IFNg+) response of peripheral blood mononuclear cells (PBMC) against autologous normal liver tissue and autologous tumor tissue in patients with secondary neoplasms of the liver of colorectal cancer origin. In addition, we measured the cytolytic activity of isolated peripheral mononuclear cells (PBMC) against homonymous colorectal cancer cells (CaCo).

Table 1 Age, sex, segmental location and number of metastases in the studied patients

No.	Age in years	Sex	Involved liver segments	Number of metastases
1	68	F	7, 8	2
2	64	F	6	1
3	63	F	6, 7	2
4	72	F	5	1
5	85	F	7	2
6	59	F	4	1
7	62	F	8	2
8	61	M	8	1
9	68	M	4	1
10	67	M	8	1
11	65	M	1	1

Materials and methods

The current prospective study was approved by the institutional board review and was performed in accordance with the Declaration of Helsinki. All patients enrolled in the current study signed an informed consent form after detailed explanation of the procedures. The consent extended to patient acceptance of LITT, possible complications and risks, biopsy procedures, blood sampling and the use of the data obtained in both the present and future studies on the condition that anonymity was maintained. All treated colorectal cancer metastases were confirmed histologically prior to therapy.

Patients

Eleven consecutive patients with liver metastases from CRC (7 female, 4 male) who met the inclusion criteria were enrolled. Mean patient age was 66.7 years (range, 59–85; SD = 7.1). The inclusion criteria for the current study were: patients with liver metastases of CRC origin; five or less liver metastases; the largest metastases being less than 5 cm in maximum diameter; contraindication to, or refusal of surgery; no extrahepatic spread and no other previous or concurrent treatment for metastatic growth.

Five patients had liver metastases of rectal cancer, six patients of colonic cancer. Seven patients had one metastasis in the liver, and four patients had two metastases. Table 1 summarizes the age and sex of the 11 patients as well as the segmental location and number of ablated liver metastases.

Biopsy and laser-induced thermotherapy technique

Liver biopsies of nontumorous (normal liver tissue) and tumorous tissue (colorectal cancer liver metastases) were obtained from every patient directly before LITT under CT guidance using an 18-G biopsy needle (Biopsy Handy, SOMATEX, Berlin, Germany).

The entire LITT treatment (done by two interventional radiologists; T.J.V. and M.G.M., with 10 years of experience in this method) was performed with intravenously injected analgesics (Pethidin 10–80 mg) and antibiosis (Cefuroxim 1,500 mg). The lesions were located on CT scans (Somatom plus 4, Siemens, Erlangen, Germany) and the injection site was infiltrated with local anesthetic (1% lidocaine 20–30 mL). Using a laser application kit (SOMATEX, Berlin, Germany) a 7 F (conventional system) or 9 F (internally cooled system) sheath was inserted under CT guidance according to Seldinger's technique. Patients were then transferred to the MR imaging unit, where MR imaging-guided ablation was performed with a Nd:YAG-laser (Dornier MediLAS 5100, Dornier MediLAS 5060, Dornier, Germening, Germany) with a wavelength of 1,064 nm, while T1-weighted gradient-echo MR imaging was performed for almost real-time thermometry. After the procedure the puncture tract was closed with fibrin glue (Tissucol Duo S; Baxter, Unterschleissheim, Germany). LITT was performed with MR imaging guidance using a 0.5-T MR imager (Privilig; Elscint, Hefa, Israel) with two T1-weighted gradient-echo MR sequences (140/12, flip angle of 80°, matrix of 128 × 256, five sections, 8-mm section thickness, 30% intersection gap, 15 s acquisition time) in transverse orientation and parallel to the laser applicators. These sequences were repeated every minute. T1-weighted thermal sequences were performed to monitor the LITT procedure in all patients. These were used in all cases to determine the duration of ablation. The ablation end-point was defined as the time point of signal loss in the thermosensitive gradient-echo sequences.

In no case was the ablation procedure performed on the basis of a predefined time or energy level. Because the heat

dispersal pattern in the tissue cannot be predicted, a given amount of energy can result in completely different volumes of coagulation necrosis. The volume of the lesion was assessed before and after LITT based on the three greatest dimensions (x, y, z), using the formula for ellipsoid volumes ($xXyXzX \times 0.523$).

IFNg secretion assay and lymphocyte staining

IFNg secretion assay and lymphocyte staining were carried out using blood and autologous test antigen obtained by liver biopsy from the same patient.

Heparinized blood (LI-Heparin 10 mL) was obtained from each treated patient before and after LITT. The samples were stored at 4° and tests performed <24 h after sampling. The biopsies obtained from each treated patient were stored at −20°C. Tissue lysates were freshly prepared in cold phosphate buffer (50 mmol/L, pH 7.2) using a glass homogenizer on ice. The suspension was filtered with a filter tip (pore size 1.2 mm) to adjust the fragment size to less than 1.2 mm. The protein concentration was measured photometrically according to Bradford and adjusted to 1 mg/mL, followed by sterilization at 600 Gy.

Autologous test antigens (normal liver tissue as well as tumor lysate from each treated patient, 12.5 mg) were added to 250 µL heparinized blood and cultured in a 15 mL conical polypropylene tube for 16 h at 37°C under 50 mL/L CO_2. A negative control without addition of antigen lysate was included, while staphylococcal enterotoxin B served as positive control antigen. Thereafter, the samples were put on ice and washed with ice cold washing solution [phosphate buffer saline (PBS, Biochrome, Berlin, Germany) containing 0.5% bovine serum albumin and 2 mmol/L ethylenediamintetraacetate (EDTA), pH 7.4] and the cell suspension was centrifuged at 300g for 10 min at 4°C. The cell pellet was resuspended with 80 mL cold culture medium Rosswell Park Memorial Institute medium (RPMI 1640 containing 10% human AB serum). "Catch" reagent (20 µL) containing a bivalent CD45 capture and IFNg binding antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) was added. The suspension was kept for 5 min on ice and after adding 5 mL culture medium the cells were incubated in closed roller tubes for 45 min at 37°C. Thereafter, 20 µL phycoerythrin (PE) labelled IFNg detection antibodies diluted 1:5 as well as 10 µL fluorescein-thiocyanate (FITC) labelled anti-CD 3, anti-CD 4 or anti-CD8 antibodies diluted 1:400 (both from Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added to the cooled and washed cells and incubated for 10 min on ice. Five microliter erythrocyte lysis buffer, containing 0.155 mol/L NH_4Cl, 10 mmol/L $KHCO_3$ and 0.1 mmol/L EDTA diluted 1:10, was added for 10 min and the cells were centrifuged at 300g for 10 min. The washed cells were resuspended in 500 µL cold washing buffer and immediately analyzed in a flow cytometer after addition of 0.25 µg propidium iodide in 5 µL distilled water. FACS data were evaluated using WinMDI Version 2.8 (free version by Joseph Trotter).

T cell conservation PBMC containing T cells were isolated in Leukosep separation tubes by density-gradient centrifugation (PAA Laboratories GmbH, Vienna, Austria). After repeated washing with PBS (Biochrom, Berlin, Germany) containing 50,000 IU/L heparin (Liquemin N 25 000; Roche, Grenzach-Whylen, Germany), cells were adjusted to a concentration of 10^6 cells/mL and cultured in RPMI 1640 (Biochrom) containing 10% AB serum (heat-inactivated and sterilized at 600 Gy), 5% HEPES buffer (Biochrom, Berlin, Germany), and 1 mg/mL penicillin/streptomycin.

For further experiments 5 Mio PBMC were resuspended in 1.8 mL cold freezing medium, containing 80% AB serum and 20% dimethylsulfoxide (DMSO), and stored at −80°C. After 24 h cells were transferred into liquid nitrogen for long term storage.

For revitalization, cells were defrosted in a water bath at 37°C and were immediately diluted in warm culture medium [RPMI 1640 (Biochrom) containing 10% AB serum (heat-inactivated and sterilized), 5% HEPES buffer, and 1 mg/mL penicillin/streptomycin].

Cytotoxicity assay

The human colorectal cancer cell line CaCo was used as a target to test for cytolytic/cytotoxic activity. All cell lines were HLA matched (ABO-system) and tested before. The cells were cultured in Dulbecco's MEM Eagle's medium (Biochrom, Berlin, Germany) with 10% fetal calf serum and 1% penicillin/streptomycin at 37°C and 5% CO_2.

Cytolytic activity of T cells was measured by an adenylate kinase (AK) release assay. Ten thousand target cells were incubated with 1,000 effector cells in a final volume of 200 mL fetal calf serum growth medium (FCS-GM) in round-bottom 96-well microtiter plates. After incubation for 4 h at 37°C, 100 mL of supernatant were harvested and stored at −20°C for further analysis.

The human colorectal cancer cell line CaCo served as a target for the patients suffering from colonic cancer metastases.

Maximum AK release was obtained by incubating target and effector cells with 1% v/v Triton-X-100, and baseline AK release with medium alone. Baseline release from T cells and tumor cells was <10% of maximal release in all experiments and subtracted from each value. The activity of AK was determined by detection of autoluminescence using a luciferase assay (ToxiLight Kit, Cambrex Corporation, NJ, USA). Twenty milliliter of supernatant were incubated with AK detection reagent (Cambrex Corporation,

NJ, USA) for 5 min at room temperature. The bioluminescence was measured using a luminometer (BD Monolight 3096 Microplate Luminometer, BD Biosciences, Heidelberg, Germany) and expressed as relative luminescence units (RLU).

Statistical analysis

The stimulation index of CD3+, CD4+ and CD8+ T cells (SI) was calculated as the ratio of IFNg+, CD3+, CD4+ and CD8+ T cells stimulated with tumorlysate versus IFNg+ T cells stimulated with autologous liver tissue lysate, respectively, using Excel® 2000 software (Microsoft, Seattle, USA). The SI for T cells stimulated with liver tissue was calculated as the ratio of IFNg+ CD3+, CD4+ and CD8+ T cells versus unstimulated IFNg+ CD3+, CD4+ and CD8+ T cells to determine unspecific immune stimulation. Results were analyzed statistically using the SPSS® software package (Version 11.01 d, SPSS Inc., Chicago, USA). SI values were calculated numerically and are presented as box-whisker plots. The significance of the enhanced SI after LITT treatment was tested with the Wilcoxon test for dependent samples. A P value less than 0.05 was considered significant.

Results

The overall pre-ablation metastasis volume (obtained by the addition of the volumes of the individual metastases in a single patient) ranged 0.5–32.6 mL (mean = 9, SD = 10.9). The total energy used during the ablation process varied from 40.8–265.9 kJ (mean = 104, SD = 66.65). The mean duration of ablation was 20 min (range 2–55 min).

In the 24 h MRI study following LITT, the ablated volume was apparently larger than the original metastatic volume, ranging from 11.5–157.6 mL (mean = 66.3, SD = 44.7) with a significant increase in volume following laser ablation.

Table 2 summarizes the changes in volume following LITT with respect to the pre-ablation volume as well as the amount of energy used during the Laser ablation procedure.

Cytokine secretion and capture assay

The test showed that autologous nontumorous control liver tissue (obtained by liver biopsy from the each patient) did not stimulate IFNg secretion of circulating T cells before or after LITT [before LITT: $SI_{[CD3+]}$ of 0.012 (±0.003), $SI_{[CD4+]}$ of 0.005 (±0.001) and $SI_{[CD8+]}$ of 0.008 (±0.002); after LITT: $SI_{[CD3+]}$ of 0.009 (±0.003), $SI_{[CD4+]}$ of 0.003 (±0.001) and $SI_{[CD8+]}$ of 0.005 (±0.003)].

When exposed to autologous tumor tissue (obtained by liver biopsy from each patient) all patients together displayed a mean $SI_{[CD3+]}$ of 12.73 (±4.83), $SI_{[CD4+]}$ of 4.36 (±3.32) and $SI_{[CD8+]}$ of 3.64 (±1.77) before LITT. Four weeks after LITT SI against tumor tissue increased to 92.09 (±12.04) for CD3+ ($P < 0.001$), 42.82 (±16.68) for CD4+ ($P < 0.001$) and 47.54 (±15.68) for CD8+ T cells ($P < 0.001$) (Fig. 1).

Table 2 Pre- and post-laser ablation volumes and the amount of energy used during ablation

No.	Pre-ablation volume (mL)	24-h post-ablation volume (mL)	Ablation energy (kJ)
1	32.6	157.6	265.9
2	6.8	53.7	104
3	26.5	114	172
4	3.8	94.9	98
5	12.1	81.4	85.9
6	0.5	18.5	40.9
7	9.8	74.4	128.5
8	1.7	11.5	40.8
9	0.5	35.4	77.5
10	1.69	22.9	85.7
11	3	64.7	44.9

Cytotoxicity assay

Cytolytic activity of T cells increased from RLU = 1,493 (±1,954.68) before LITT to RLU = 7,260 [±3,929.76 ($P < 0.001$)] after LITT (Fig. 2).

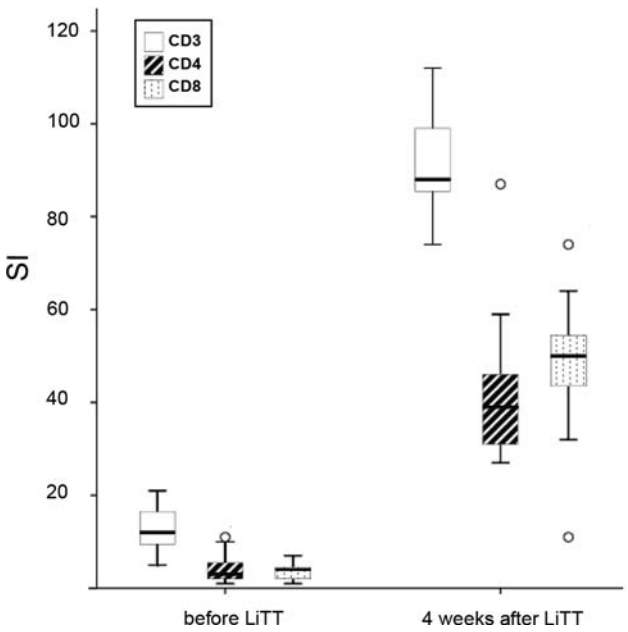

Fig. 1 Stimulation index (SI) of isolated CD 3, 4, 8 lymphocytes after re-exposure to autologous tumor tissue. Activation was measured by CD45 capture for interferon gamma and double staining for CD3, 4, 8, response. Outtakes are marked with *open circle*

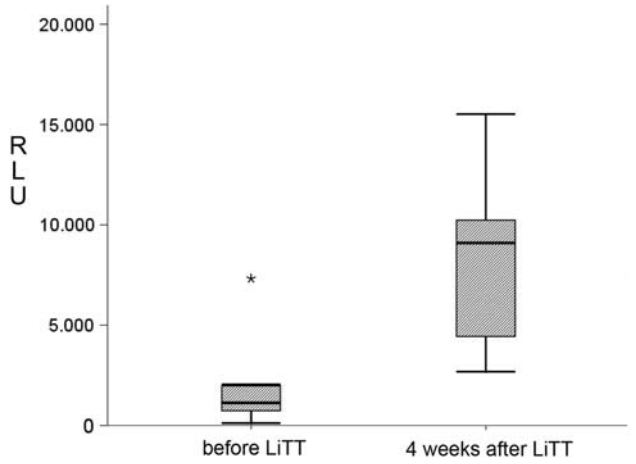

Fig. 2 Cytotoxic activity before and 4 weeks after LITT. Release of cytoplasmatic adenylate kinase of homonymous tumor cells (CaCo) after co-incubation of isolated PBMC before and 4 weeks after LITT. Four weeks after LITT there is a significant upregulation of cytotoxic activity of isolated PBMC of treated patients. Extremes are marked with *asterisk*

Discussion

Thermal ablation methods such as RFA or LITT are increasingly used in the therapy of unresectable liver tumors [8–14]. Vogl et al. [2] reported survival rates of 94% after 1 year, 77% after 2 years, 56% after 3 years and 37% after 5 years in patients treated with LITT for liver metastases of colorectal cancer origin. Solbiati et al. [3] reported survival rates of 93% after 1 year, 69% after 2 years and 45% after 3 years for patients with liver metastases of colorectal cancer origin treated with radiofrequency ablation. Surgery remains the gold standard treatment of liver metastases. Nevertheless, data from thermoablation procedures are comparable with results of surgical resection, which have been reported as 89–93% after 1 year, 71% after 2 years, 57% after 3 years and 29–40% after 5 years for patients with liver metastases of colorectal cancer [15–17].

The first reports on the use of laser energy to ablate liver metastases appeared in the early 1990s [18–20]. The main advantage of LITT compared to surgical resection in the treatment of liver tumors is that it is a minimally invasive technique. Furthermore, it allows treatment of unresectable tumors in selected patients [21]. A further major advantage of MR imaging-guided LITT is that it can be performed with local anesthesia in an outpatient setting; the complication rate is low [2].

The extent and completeness of LITT-induced tumor necrosis depends on a series of complex cellular and molecular events that are influenced by various interrelated factors [22]. Therefore, it appears improbable that the achieved results can merely be explained by physical effects like thermal tumor ablation or by a favorable patient selection in the case of the nonsurgical group. Patients treated with RFA are more likely to show a negative-outcome bias, since they are often not eligible for hepatic resection due to poor liver function or advanced tumor disease.

Immunotherapy of solid tumors has moved more and more into the focus of attention in the last decade. Great efforts have been directed towards the field of T cell based immunotherapy by dendritic cell vaccination, e.g., for patients suffering from melanoma. Local treatment of solid tumors by cytokines has also produced promising results. Most of these therapies lack tumor antigens or are very cumbersome.

It is well known that RFA and LITT can induce an unspecific immune stimulation, as thermal coagulation causes an inflammatory reaction with lymphoplasmacellular infiltration that can be visualized as a hypervascular rim in contrast-enhanced CT and contrast-enhanced ultrasound.

Several previous studies have shown evidence of immunological responses to RFA [4, 5, 23, 24]. They have also shown that besides reducing tumor mass, RFA provokes an adjuvant immunological response. There are also reports of significantly elevated levels of tumor-specific PBMC in animals and of tumor-specific T cells in patients.

There is evidence from transplanted syngenic tumor models in rats that LITT has an immunologic effect [6, 7].

The aim of this study was to assess whether a similar immunological response is observable also in patients after laser-induced thermal ablation.

In the present preliminary study we performed two different assays:

(a) An IFNg secretion assay and lymphocyte staining using blood and autologous test antigen obtained by liver biopsy from each individual patient, (b) a cytotoxicity assay, using an allogenic cell line (CaCo).

For the IFNg secretion assay and lymphocyte staining we used only autologous test antigen obtained by liver biopsy from each individual patient to rule out any immunological reaction to allogenic antigen. We were able to demonstrate that patients with colorectal cancer metastases have significantly elevated peripheral tumor-specific IFNg+, CD3+ T cells, CD4+ T helper cells and CD8+ T killer 4 weeks also after LITT.

The cytotoxicity assay showed an increase of the cytotoxic activity of the T cells derived from PBMC over baseline 4 weeks after LITT. Since an allogenic cell line (CaCo) was used as a target for the cytotoxicity assay an immunological reaction towards the allogenic antigen cannot be ruled out in this test. Further studies investigating the influence of LITT on the cytotoxic activity of T cells using autologous primary tumor cell cultures are needed. In this study, all the autologous tissue obtained from each patient by biopsy had to be used for the cytokine secretion and capture assay. For ethical reasons, the number of biopsies was

strictly limited to one biopsy from the tumor and one biopsy from the nontumorous liver. An interesting task for future research will be to determine whether this effect is as strong in primary cell cultures as it was in these allogenic cells.

As RFA and LITT both result in tumor-specific immune stimulation, it can be assumed that the decisive factor for the immune response is the thermal destruction of the tumor cells and that this response does not depend on whether the thermal destruction was brought about by radiofrequency waves or laser energy.

Both methods appear to present tumor-specific antigens in an appropriate manner in order to activate tumor-specific T cells and raise cytolytic activity.

Thus, it appears that the coagulation of tumor tissue through RFA or LITT leads to the enhanced release, exposure and/or denaturation of tumor antigens. Thermally altered tumor antigens are more likely to be phagocytosed by professional antigen presenting cells like dendritic cells. In conjunction with the generation of thermally altered tumor antigens, the unspecific inflammatory stimulus induced by RFA or LITT might help to overcome immunetolerance or anergy towards the tumor. The pro-inflammatory effects of necrotic cells are well documented and appear to be caused by the release of endogenous adjuvants such as the nuclear protein high mobility group B1 (HMGB1) or heat shock proteins, e.g., hsp70 or gp96. RFA and LITT thus appear to create an in situ environment resembling T cell vaccination ex vivo.

Based on the current study, it can be assumed that the immune system at least contributes partially to the effectiveness of LITT in the control of liver metastases of colorectal cancer origin. Further work is still required, including research work to compare the extent of immunologic response following RFA and LITT as well as clinical work in terms of the introduction of new drugs such as TLR stimulators. These may have the potential to further enhance the immune stimulation caused by thermal ablation. It should also be worthwhile to test these combination therapies in further trials.

Limitations to the current study are the inclusion of only patients with liver metastases originating from colorectal cancer and the relatively small number of patients. However, enrolling a larger number of patients would have posed other problems, given the need for timely coordinated work between two departments and, more importantly, the invasive nature and added risk of the liver biopsy.

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