

Regulatory T cells in children undergoing rush venom immunotherapy

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ABSTRACT

Venom immunotherapy (VIT) induces immune tolerance to Hymenoptera venom but the underlying mechanisms are not clarified. Regulatory T cells are thought to play an important role in tolerance induction during specific immunotherapy. Our objective was to determine the effects of rush VIT on the percentage of regulatory T cells and immunosuppressive cytokines interleukin (IL)-10 and transforming growth factor beta (TGF- β) in children. Blood samples were collected from 18 children with a previous systemic allergic reaction to a Hymenoptera sting, with a positive skin test and positive specific IgE, before rush VIT, after 6 weeks and 6 months of rush VIT. Ten children with no history of venom allergy were studied as controls. Isolated peripheral blood mononuclear cells were stained with specific markers for regulatory T cells and analyzed by flow cytometry. The percentage of regulatory T cells did not change during rush VIT in children. No change was noticed in the percentage of IL-10 and TGF- β secreting cells after 6 weeks or 6 months of VIT. No difference in expression of cytotoxic T-lymphocyte antigen 4 on CD4⁺CD25^{high} was found. Rush VIT is a safe and effective treatment for patients allergic to Hymenoptera venom. Although regulatory T cells are considered to be responsible for this effect; no significant changes in the percentage of these cells or immunosuppressive cytokines were noticed during rush VIT in children. Additional investigations are needed to clarify the role of regulatory T cells in the induction of tolerance during rush VIT in children.

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Insect stings present an exposure to a relatively high systemic dose of venom but are relatively infrequent as opposed to the chronic exposure of inhalational allergens. Allergic reactions to insect stings can be life-threatening. Venom immunotherapy (VIT) is the only therapy that provides long-term tolerance to the patient with a high risk of sting-induced anaphylaxis. The efficiency of VIT is well documented.^{1–5} Successful immunotherapy is associated with an increase of IgG antibodies, especially of the IgG4 class, which are considered as allergen blockers.^{6–8} Some studies have shown an increase of interferon (IFN) γ and a decrease of interleukin (IL)-4, as well as a reduction in mastocyte and eosinophile numbers, and other studies have shown a decrease in IL-4 and IFN- γ .^{9–12} This anergic response was explained as caused by the increase of the immunosuppressive cytokine IL-10 after successful immunotherapy.^{13–18} IL-10 initiates anergy by blocking the CD28 costimulatory signal.¹³ Type 1 regulatory T cells (Tr1) are defined by their ability to secrete IL-10 and are considered to be a source of increased

IL-10 during immunotherapy.^{17,18} An increase in natural occurring regulatory T cells (CD4⁺CD25^{high}) and immunosuppressive cytokines (IL-10 and transforming growth factor [TGF] β) was reported in patients undergoing house-dust mite and grass pollen immunotherapy.^{18,19} Despite all of the described changes, the exact mechanism of immunotherapy is still not clear. In recent studies on adult patients, an increase of naturally occurring regulatory T cells CD4⁺CD25^{high} has been observed in the earliest phase of rush VIT.²⁰ In this study we sought to evaluate the percentage of regulatory T cells and T cells that secrete immunosuppressive cytokines (IL-10 and TGF- β) in children during the first 6 months of rush VIT.

METHODS

Patients

This uncontrolled open-label study included 18 children (9 boys and 9 girls) aged 7–15 years (mean age, 9 years) with a personal history of IgE-mediated systemic allergic reaction to Hymenoptera stings, grades III and IV, according to Mueller.²¹ The patients' histories of symptoms after an insect sting were evaluated in a standardized questionnaire. IgE-mediated allergic reactions were confirmed by skin testing and elevated serum titers of venom-specific IgE (sIgE) antibodies. Inclusion criteria were based on the European Academy of Allergy and Clinical Immunology position paper.²² Eleven patients were allergic to bee venom, one patient to yellow jacket venom, and six patients

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were allergic both to bee and to yellow jacket venom. Skin tests and sIgE serum values were assessed before treatment and sIgE values were measured after 6 weeks and 6 months of rush VIT. Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral blood by means of centrifugation over Ficoll-Hypaque gradient (Pharmacia, Uppsala, Sweden) before, after 6 weeks, and after 6 months of rush VIT. Isolated PBMCs were stored in liquid nitrogen and analyzed from the three time points at once.

Skin Test

Skin-prick tests were performed with standardized pure venom extracts of the honeybee and *Vespula* spp. (Stallergenes, Antony, France). Histamine dihydrochloride (10 mg/mL) and glycerol diluent were used as positive and negative controls, respectively. The prick test in double-positive patients was followed by an intradermal test on the forearm with increasing concentrations from 0.001 to 1 μ g/mL. Intradermal tests were considered positive if reactions (wheal of at least 5 mm in diameter with erythema) occurred after 15 minutes at a concentration of ≤ 1 μ g/mL.

Specific IgE

In all patients, venom sIgE antibodies to honey bee and yellow jacket were determined by using a fluorescence immunoassay (CAP; Pharmacia). The analysis was performed by a UniCAP 100 analyzer (Phadia, Uppsala, Sweden). Results were calculated in kilounits per liter for sIgE antibodies. All sIgE antibody values of ≥ 0.35 kU/L were considered positive.

Rush VIT

The rush VIT regimen was completed within 5–7 days while the patients were hospitalized. The protocol began without premedication, with an initial dose of aqueous extract, gradually increasing subsequent subcutaneously administered doses at intervals of 60 minutes, until the maintenance dose of 100 μ g (1 mL) was reached. Booster injections of 100 μ g were given at 7, 14, and 21 days after the completion of the rush schedule. Thereafter, the patients received monthly injections of 100 μ g of bee or *Vespula* spp. (Stallergenes) over a period of at least 6 months. Samples of heparinized blood and serum were obtained before immunotherapy, after 6 weeks, and 6 months of initiation of immunotherapy. The control group consisted of 10 children (6 boys and 4 girls) aged 8–12 years (mean age, 9 years) free of manifest allergic disorders and with a negative family history of atopy. The study was approved by the medical Ethics Committee of Children's Hospital Srebrnjak.

Monoclonal Antibodies

For phenotype analysis using flow cytometry, we used fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAbs) to CD4; phycoerythrin (PE)-conjugated mAbs to cytotoxic T-lymphocyte antigen 4 (CTLA-4), CD45RO, CD69, and HLA-DR; CyChrome-conjugated CD25 mAb (all from BD Biosciences, Heidelberg, Germany); allophycocyanin (APC)-conjugated anti-CD3 (Serotec, Oxford, U.K.), PE-conjugated mAbs to IL-10 (BD Biosciences); and TGF- β -1 (IQ Products, Groningen, The Netherlands).

The standard procedure of four-color direct immunofluorescence staining of PBMCs was performed according to the manufacturer's protocol. The following combinations of mAbs were used: CD4 FITC/CTLA-4 PE/CD25 CyChrome/CD3 APC, CD4 FITC/CD69 PE/CD25 CyChrome/CD3 APC, CD4 FITC/CD45RO PE/CD25 CyChrome/CD3 APC, and CD4 FITC/HLA-DR PE/CD25 CyChrome/CD3 APC. For CTLA-4 determination, cells were stained with anti-CD3, anti-CD4, and anti-CD25; fixed with 4% formaldehyde in phosphate-buffered saline (PBS); and then permeabilized with 0.1% saponin (Sigma-Aldrich Corp., St. Louis, MO) in PBS before the addition of PE-conjugated anti-CTLA-4. CD3⁺CD4⁺ helper T lymphocytes (gated from scatter graph based on size and coexpression of CD3 and CD4) double positive for CD4 and CD25^{high} were designated as Treg cells as described previously.²¹ For each sample, at least 5000 CD4⁺CD25^{high} events were collected and analyzed for the coexpression of CD69, CD45RO, HLA-DR, CTLA-4 using a dual-laser, four-color FACSCalibur flow cytometer, and CellQuest software or a BD LSR II flow cytometer using FACS Diva software (BD Biosciences).

Intracellular Cytokine Staining

For intracellular detection of TGF- β 1 and IL-10, PBMCs were cultured in RPMI-1640 supplemented with 10% heat-inactivated AB serum and antibiotics alone or in the presence of 50 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich Corp.) and 0.75 μ g/mL of ionomycin (Sigma-Aldrich Corp.) as described previously.²³ Brefeldin A (10 μ g/mL; Sigma-Aldrich Corp.) was added for the last 6 hours of incubation. For the last 15 minutes, 20 mmol/L of EDTA was added. The PBMCs were stained with CD3-FITC and CD8-PerCP, washed, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.1% saponin in PBS, and then PE-conjugated anti-IL-10 or anti-TGF- β 1 was added and incubated for an additional 30 minutes at 4°C. CD3⁺CD8[−] cells were considered to be CD4 cells because phorbol 12-myristate 13-acetate and ionomycin stimulation results in internalization of CD4.²⁴ Samples from patients and their age- and sex-matched controls were analyzed after 24 hours of cell culture.

Statistical Analysis

Because of the non-Gaussian distribution and the small sample size, the nonparametric Mann-Whitney test was used for the study and control group comparison. Friedman analysis of variance followed by multiple comparisons using Wilcoxon tests were used to detect changes within study groups during immunotherapy. The relationship between variables was evaluated by means of the Spearman rank-order coefficient. We used a statistical software package (Statistica Version 6.0; Statsoft, Inc., Tulsa, OK) and considered the probability significant at $p < 0.05$.

RESULTS

Eighteen children with severe systemic reaction to Hymenoptera venom, grades III–IV according to Mueller's classification underwent rush VIT.²¹ Six patients experienced re-stings during this study, five patients had no reaction or a small local reaction, and just one patient who had a grade IV pretreatment reaction had a grade I reaction after spontaneous re-sting.

Specific and total IgE levels were determined before, after 6 weeks, and after 6 months of immunotherapy. Significant decrease of sIgE was noticed after 6 weeks of VIT ($p = 0.04$). Further decrease was measured after 6 months of immunotherapy ($p = 0.01$; Fig. 1). Total IgE did not significantly change during VIT.

CD4⁺CD25⁺high during Venom Rush Immunotherapy

Previous studies have shown that only CD4 T cells with a highest CD25 expression have a suppressive activity. Gating strategy, as previously described for detection of CD4⁺CD25⁺high was used.^{23–26} To exclude activated cells, marker of activation CD69 was analyzed on these cells.

The proportion of CD4⁺CD25⁺high cells did not change significantly after 6 weeks and 6 months of immunotherapy (Fig. 2 A). There was no difference in expression of CD45RO and HLA-DR on the surface of these cells. Intracellular expression of CTLA-4 on these cells was measured and no difference in expression of CTLA-4 on CD4⁺CD25⁺high after 6 months of immunotherapy was found (Fig. 2 B). No difference in frequency of CD4⁺CD25⁺high between healthy children and children with previous severe allergic reactions to insect stings was found. There was no difference in expression of CTLA-4 on CD4⁺CD25⁺high between allergic and healthy children.

Cytokine Production during Rush Immunotherapy

The effect of VIT on IL-10 and TGF- β 1 secreting T cells was determined before and after 6 weeks and six months of immunotherapy. No statistical significant difference in percentage of TGF- β 1 and IL-10-secreting

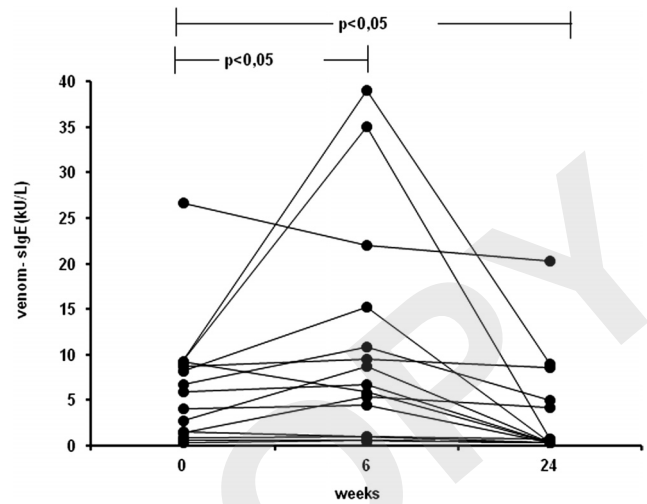


Figure 1. Serum level of venom-specific IgE (sIgE) during rush venom immunotherapy (VIT). Serum level of venom sIgE was analyzed in 18 patients undergoing specific rush immunotherapy before and after 6 weeks and 6 months of immunotherapy. Significant decrease of venom sIgE was noticed after 6 months of immunotherapy. Each symbol presents one participant.

T cells was found after 6 weeks and 6 months of VIT (Fig. 3, A and B). Although an increase in percentage of IL-10-secreting cells was noticed in 10 children after 24 weeks, no statistical significance was reached (Fig. 3 B). There was no difference in percentage of IL-10 and TGF- β 1-secreting T cells between allergic children during rush VIT and healthy children.

DISCUSSION

VIT is established as a highly effective, specific form of treatment to prevent life-threatening reactions in Hymenoptera allergies. The goals of VIT are to reach an allergen dose, inducing tolerance to Hymenoptera venom, with the lowest rate of systemic reactions. The underlying immunologic mechanisms of VIT are continuously being elucidated. Different mechanisms involving specific cell populations play a role in the different phases of VIT, even though some of the described events may represent epiphenomena rather than causative events.²² Decrease of sIgE occurs during immunotherapy. Disappearance of skin test and sIgE indicates clinical protection. We found significant decreases in sIgE levels after 6 weeks as well as after 6 months of VIT when compared with baseline levels. There is evidence that regulatory T cells may suppress sIgE production and increase sIgG4 *via* IL-10, which is induced and progressively secreted during successful rush VIT.¹⁶

Although many studies showed an implication of regulatory T cells in allergic disease, to our knowledge this is the first study of CD4⁺CD25⁺high regulatory T cells during rush VIT on a population of children. We

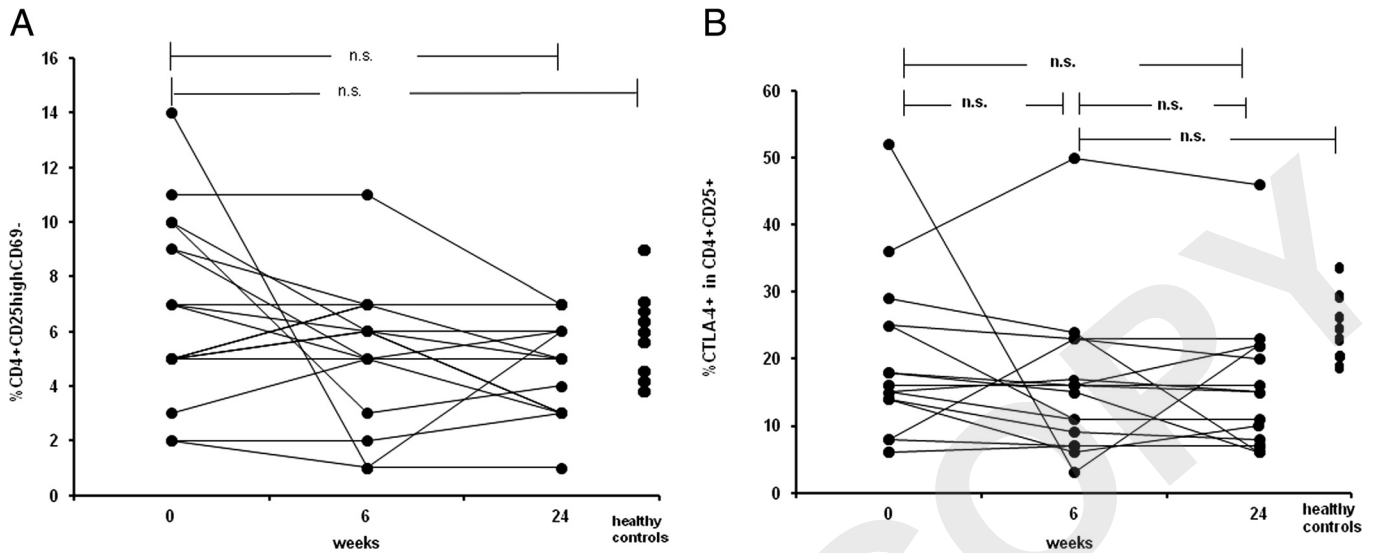


Figure 2. (A and B) Percentage of CD4⁺CD25^{high}CD69⁻ and CD4⁺25⁺CTLA-4⁺ cells during rush venom immunotherapy (VIT). Percentage of CD4⁺CD25^{high}CD69⁻ and CD4⁺25⁺CTLA-4⁺ cells in healthy children and children allergic to Hymenoptera venom undergoing specific rush immunotherapy. Data from 18 patients were analyzed before and after 6 weeks and 6 months of immunotherapy. No significant changes in percentage of (A) CD4⁺CD25^{high}CD69⁻ or (B) CD4⁺25⁺CTLA-4⁺ cells were observed after 6 weeks and 6 months of immunotherapy. Each symbol presents one participant; n.s., nonsignificant.

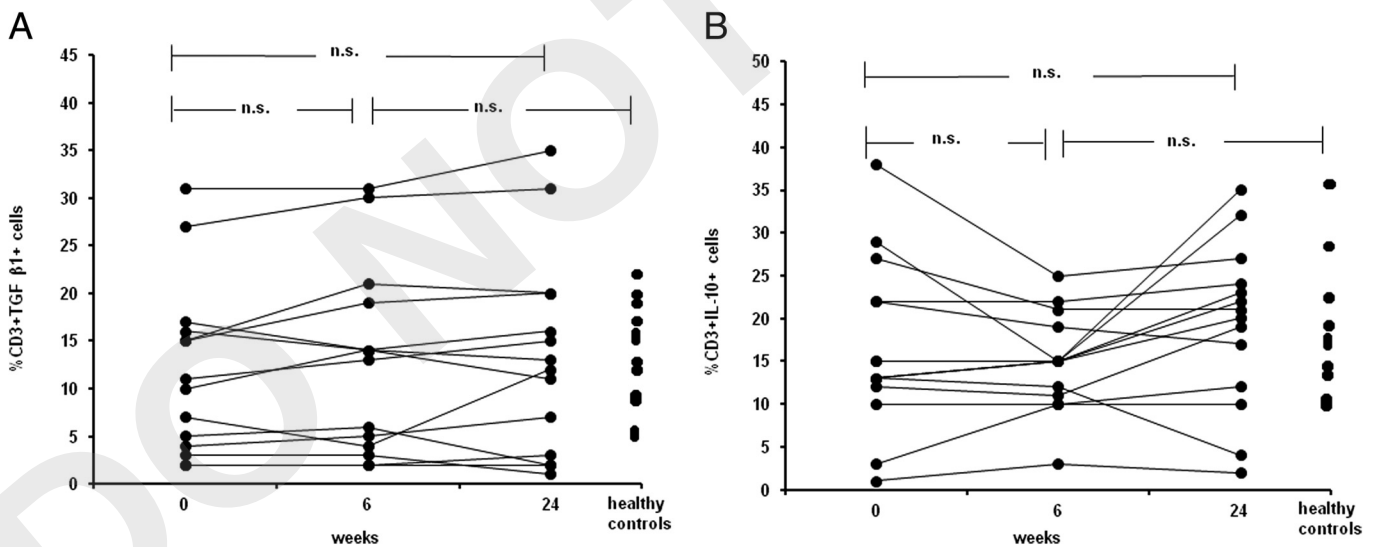


Figure 3. (A and B) Percentage of CD3⁺CD8⁻TGFβ1⁺ and CD3⁺CD8⁻IL-10⁺ cells during rush venom immunotherapy (VIT). Percentage of CD3⁺CD8⁻TGFβ1⁺ and CD3⁺CD8⁻IL-10⁺ cells in healthy children and children allergic to Hymenoptera venom undergoing rush immunotherapy. Isolated polymorphonuclear blood cells were stimulated with phorbol 12-myristate 13-acetate and ionomycin for 24 hours. Levels of interleukin (IL)-10 and transforming growth factor (TGF) β1 were determined by intracellular cytokine staining. There was no difference in percentage of (A) CD3⁺CD8⁻TGF-β1⁺ or (B) CD3⁺CD8⁻IL-10⁺ cells before or after 6 weeks and 6 months of immunotherapy. Each symbol presents one participant; n.s., nonsignificant.

found no statistical significant change in percentage of CD4⁺CD25^{high} regulatory T cells after 6 weeks and after 6 months of rush VIT. An increase of CD4⁺CD25^{high} was noticed in adult patients after 6 months of immunotherapy.²⁷ Other studies have shown an increase in the percentage of these cells after 1 day of rush VIT; however, these changes were described in adult patients with previous mild allergic

reactions.¹⁸ As mentioned before, all of the children in our study had a history of a severe allergic reaction to insect stings, which was graded according to Mueller class III and IV. A recent study on adult patients during standard VIT showed a small decrease in the percentage of CD4⁺CD25^{high} during the 1st month of immunotherapy.²⁸ Explanation for the transient decrease of Treg is a possibility of homing of Treg in

secondary organs where they suppress T-cell effector response. This could explain why we found no change in percentage of Treg in children during VIT.

We found no difference in the percentage of CD4⁺CD25^{high} between allergic and healthy children, and the study on adults showed that frequency of these cells in the peripheral blood of the patients with a severe allergic reaction to insect stings was diminished compared with healthy individuals.²⁰ Research on children with asthma and chronic cough also found no difference in percentage of CD4⁺CD25^{high} in peripheral blood compared with healthy children.²⁹ In our previous study on children allergic to house-dust mites undergoing specific immunotherapy for 1 year, we also found no changes in percentage of CD4⁺CD25^{high}.²⁶

FOXP3 has been described as an essential transcriptional factor for the development of regulatory T cells.^{30,31} Because of a limited amount of blood that could be taken from children, FOXP3 was not stained, but to be sure that CD4⁺CD25^{high} was Treg and not activated T cells, a CD69 marker was used.

Previously it was suggested that CD4⁺CD25^{high} exerts regulatory function through CTLA-4.^{32,33} We investigated the percentage of these cells and found no significant difference during the first 6 months of immunotherapy. It has been suggested that up-regulation of CD4⁺CD25⁺ Treg cells plays a role in allergen immunotherapy. Accordingly, using a rush protocol in vespid-allergic patients, an increase in CD4⁺CD25⁺ Treg cells and IL-10-producing T cells has been shown in the hours after the commencement of VIT.¹⁸ It is unclear as to why no change in the percentage of CD4⁺CD25^{high} was noticed during the first 6 months of VIT immunotherapy in children, when in some adult studies, an increase of these cells was noticed. The switch from TH2 to TH1 occurs early during rush VIT. Cytokine changes such as IL-4 decrease and IFN- γ increase occur as early as the 1st day of immunotherapy. IL-10 is considered to play an important role in immunomodulation during immunotherapy.^{16–20} A recent study during standard VIT has shown an increased production of IL-10 after 1 month and virtually baseline production levels after 6 months of VIT.²⁸ We found no statistically significant increase of IL-10 during the first 6 months of rush VIT, although a small increase of IL-10-secreting T cells was noticed in 10 children. In our research a rush VIT was conducted so it could be that an increase of IL-10-secreting cells occurs earlier, and after 6 weeks it is already decreased to a baseline production. TGF- β 1 has been described as a possible mechanism of suppressive activity of regulatory T cells.^{32–35} Suppressive activity of Treg obtained from bronchoalveolar lavage samples of allergic children with asthma was dependent on TGF- β 1.²⁹ In our research, no change in the percentage of TGF- β 1-

secreting T cells was found after the first 6 months of immunotherapy.

The responsible mechanism of a significant decrease in specific IgE after 6 weeks and during the 6 months of rush immunotherapy is still unclear, and it does not seem to be directly influenced by the activation of regulatory T cells. The clinical efficacy of VIT has been proven by several trials, but the underlying immunoregulatory mechanisms of protection remain poorly understood.

Treg cells are an important factor for induction of tolerance, as has been shown in many studies on adult patients; however, we still do not know the real mechanism on how Treg cells induce tolerance. In this study we investigated the role of Treg in children during rush VIT, but our study is limited by the small number of patients and the research on children population is difficult because of the limited amount of cells that could be isolated. Additional research on a larger number of children and earlier detection of cytokine secreting cells and Treg cells during rush VIT is required.

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