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Short Communication

Gintonin upregulates cytokine production and expression of NKp30, NKp44 and NKp44 related to natural killer cell activity on immunosuppressive rat

BaiCheng Chen [a], Ajay Vijayakumar [a], Chul Park [a], Ulsoo Choi [a], Seung-Yeol Nah [b,**], Jong-Hoon Kim [a,*]

[a] College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, Iksan-city, Jeollabuk-Do, Republic of Korea
[b] Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, Republic of Korea

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ABSTRACT

The objective of the study is to estimate the potential of gintonin, as an immune enhancing agent through natural killer cell (NK cell) activity in cyclophosphamide (CY)-induced immunosuppressive animals. Accumulated results reveals that, gintonin attenuated CY-induced immunosuppression and it might modulate NK cell activity to boost the immunity.

Ginseng has been significantly used as a medicine and tonifying agent to the body in Orient countries, especially Korea, China, and Japan. Several studies have suggested that ginseng show antioxidant, anti-inflammatory, and anticancer effects [1,2]. In the past two decades, many medicinal herbs became popular because of their safety and effectiveness in disease treatment [3]. *Panax ginseng* a popular traditional herb is considered as an medicine that can improve stamina to individuals [4]. Recently, we isolated gintonin, a novel constituent of ginseng, with about 17 kDa as a new constituent of *Panax ginseng* [5], which consists of a complex of lysophosphatidic acids, ribonuclease-like storage protein, and latex-like protein 151 [6]. Earlier, gintonin was reported to exhibit pharmacological effects on neuronal diseases such as Alzheimer's and Parkinson's diseases [7,8]. However, the effects of natural killer cell (NK cell) activity in immunosuppressive animal model, are unknown. NK cells play a crucial role in regulating the immune system against abnormal cells. NK cells undergoes lysis on tumor and virus-infected cells upon releasing perforin and granzyme that targets the abnormal cells directly. However, its function will be hindered following administration of immunosuppressive drugs. Thereby NK cell based immunotherapy are extensively studied for immune boosting, especially on herbal extracts since it is associated with low toxicity. Cyclophosphamide (CY), a chemotherapueitc drug might damage normal cells especially the immune cells and it can be used to induce immunosuppression in animal models. Previously, CP has been applied to induce suppression of immune system and examine the immune enhancing effects of the studied compound or herbal extract [9,10]. Therefore, in this study gintonin was prepared as previously decribed [11], and we studied the immunomodulatory effects of gintonin in cytokine production and NK cell activity in CY immune suppressed animals.

Six week old male Sprague-Dawley rats weighing 200 ± 20 g were purchased from SLC Inc., Japan. The Principles of Laboratory Animal Care were followed in accordance with the "Guideline for Institutional Animal Care and Use Committees" of Chonbuk National University (IACUC No.: NON2023-03-021). Animals used were randomly divided into four groups (n = 6), and allowed to acclimatize for one week. Normal group (NC) received sterile distilled water and was not immune suppressed with CY. To all other animals CY was intramuscularly injected on zero day, third day and sixth day with CY (30 mg/kg BW; Sigma–Aldrich, USA). Gintonin was dissolved in sterile distilled water and administered orally using catheter. All other chemicals were purchased from Sigma (St. Louis, MO, USA). The gintonin-treatment groups were orally administrated with 100, and 300 mg/kg gintonin respectively. Gintonin-treatment groups received the respective dose once a

* Corresponding author. College of Veterinary Medicine, Biosafety Research Institute, Chonbuk National University, 54596, Iksan-city, Jeollabuk-Do, Republic of Korea.
** Corresponding author. Ginsentology Research Laboratory and Department of Physiology, College of Veterinary Medicine, Konkuk University, Seoul, 05029, Republic of Korea.
E-mail addresses: synah@konkuk.ac.kr (S.-Y. Nah), jhkim1@jbnu.ac.kr (J.-H. Kim).

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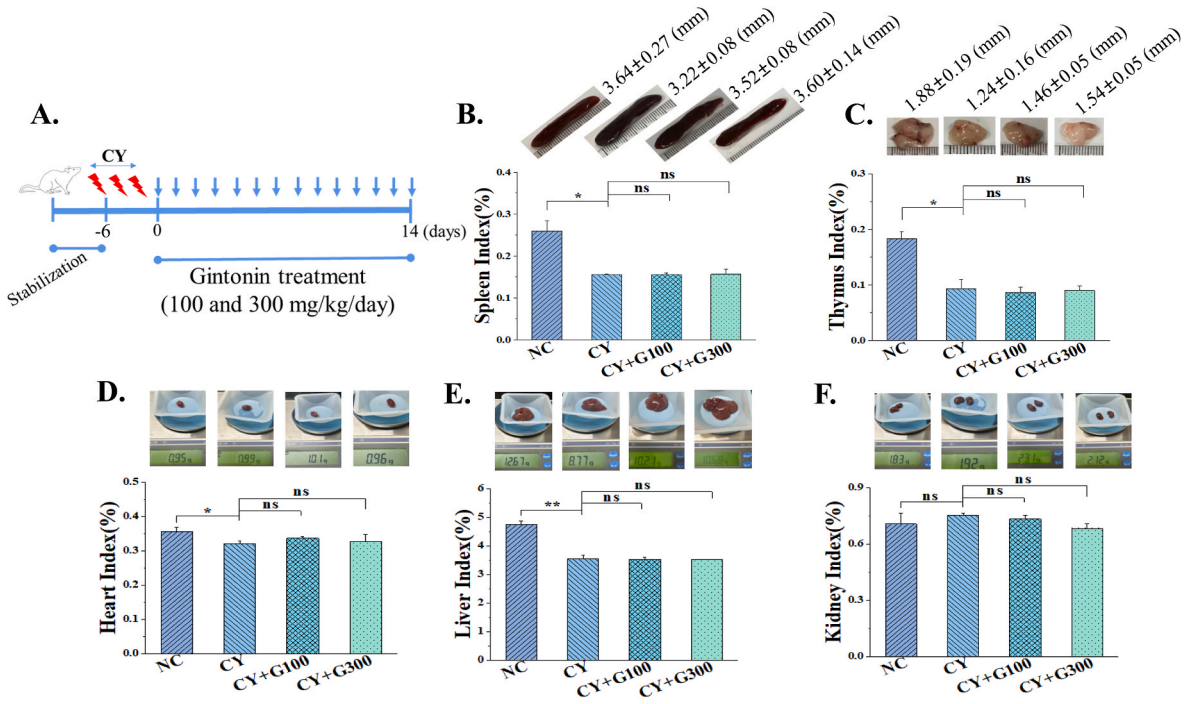

Fig. 1. Schematic diagram for the experiment (A), and organ index for spleen, thymus, heart, liver, and kidney (*P < 0.05 , **P < 0.01 , ns > 0.05 compared with the control group) (B–F). Abbreviation: NC, normal control; CY, cyclophosphamide; G, gintonin.

day for 14 consecutive days (Fig. 1A). Animals were sacrificed at the end of treatment to analyse the hematological parameters, including red blood cell (RBC), white blood cell (WBC), lymphocyte, hematocrit, and hemoglobin. Peripheral blood from rats were carefully collected into tubes containing ethylenediaminetetra-acetic acid (Becton Dickinson, Parsippany, NJ) and centrifuged at 600×*g* for 15 min, the supernatant was collected and stored at −70 °C for further analysis. The constituents were analyzed using ADVIA 2120 (Siemens Healthineers, Erlangen, Germany) according to standard procedures at the department of clinical pathology, Chonbuk National University. After sacrifice, the organs of each animal was weighed individually to calculate the organ index. [Organ index (%) = Organ weight (g)/Animal weight (g) X 100]. Among various immune cells, T cells have several subtypes, including T helper cells (type 1 and type 2), cytotoxic T cells, and effector T cells. Particularly, these T helper cells play important roles under many immune responses. Type 1 cells are activated during cell-mediated immunity, however, type 2 cells are specifically stimulated during humoral immunity [12]. T cells secrete cytokines upon activation, which in turn produce an inflammatory response to defend the host from harmful infections. T helper cells support the immune system when the cytokines are upregulated by immune response. Specially, IL-2, and IFN-γ are regulated by type 1 T helper cells, whereas cytokines such as IL-4, IL-6, and IL-10 are controlled by type 2 T helper cells [13]. The different types of cytokines were assayed using enzyme-linked immunosorbent assay (ELISA) as per the kit (BMS634, ERA29RB, BMS625, BMS621, Thermo Fisher Scientific Company, USA; ab214566, Abcam, UK). In addition, western blot was performed to analyse the protein expression related to immune activation such as perforin (NK cytolytic molecules, CD16, NKp30, and NKp44. NK cell activity was measured using flow cytometric analyses were performed with appropriate antibodies. For cell surface staining, anti-CD16, anti-CD56, PE-anti-NKG2D, PE-anti--granzyme B, anti-NKp30, and APC-anti- NKp46 were used. Accumulated data were analyzed using FlowJo software (USA). To estimate the expression of NK functional markers, equal protein amount of lysate of the splenocytes were separated by 10% SDS PAGE and transferred on membrane (Milipore, USA). Followed by treating with primary antibodies against perforin, CD16, NKp30, NKp44 and β-actin and incubated with conjugated secondary antibody (Santa Cruz Biotechnology, USA) for 1 h at room temperature. The intensity of each band was calculated by the program Analyzer (USA) and normalized to β-actin. Furthermore, we performed flow cytometry analysis to identify the effects via which gintonin increases the activity of NK cells, and the correlation of gintonin treatment with surface expression of NK activating receptors such as NKp30, and NKp46 using MoAb specific for NKp30, and NKp46 [14]. All data were subjected to one-way analysis of variance and a value of $P < 0.05$ is considererd to be statistically significant.

Organ indexes revealed that CY-treated group had significantly lower values than that in NC group and the gintonin treatmentment groups did not elicit any difference in the organ index regardless of the dosage, indicating that gintonin does not affect the organs such as spleen, thymus, heart, liver, and kidney (*p < 0.01) (Fig. 1B–F). NK cells are separated into subsets on the basis of CD56 receptor density [15], and NK cell expressed CD16 receptors and induced potent cytotoxicity against the foreign cell such as K562 [16]. Therefore, we measured the NK cell populations such as $CD16^+$ and $CD56^+$ cells in all groups, and we found that the percentages of $CD56^+$ and $CD16^+$ cell subsets were significantly increased in gintonin-treated group than those of CY group (Fig. 2A), which explains the possibility that gintonin could increase the activity of NK cells that kills abnormal cells. Flow cytometry analysis demonstrated that gintonin treatment upregulated the expression of NK activating receptors such as NKG2D and granzyme B. There was no difference between NC and CY groups (Fig. 2B), which could be because CY first causes depletion of all lymphocytes, but effector T cells mobilize in response to the depletion. Although the action of effector T cells and NK cells by CY are only limited, the expression of granzyme B by tumor-specific T cells and NK cells is upregulated to some extent [17, 18]. However, there was no dose dependent increase observed in granzyme-B marker which needs further investigation. Likewise, NKp46 expression observed only a marginal increase compared to CY group, even though the expression of NKp30 were significantly increased in gintonin-treated group than CY group (Fig. 2C). The cytokine levels in gintonin-treated group were higher than that in the CY-treated group (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) (Fig. 3A–E). IFN-γ, IL-6 exhibited a dose dependent reaction, while there was not a notable difference

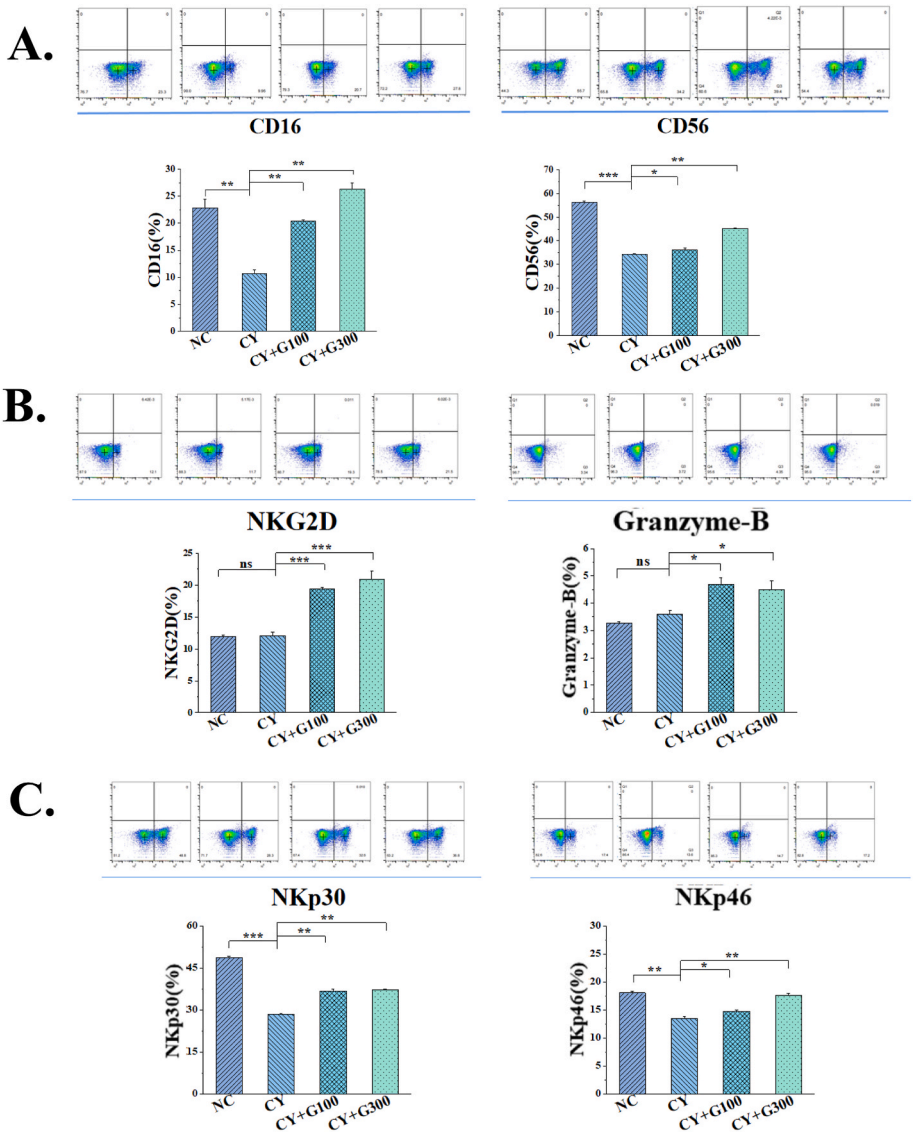

Fig. 2. Flow cytometry analyse for the proportion of NK cell surface receptors and granzyme B in splenic cells of CY-treated rats. It was shown that the treatment of gintonin increased the expression of the activation markers of NK cells (A–C). All data are expressed as the means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.01 compared with the CY group. Abbreviation: NC, normal control; CY, cyclophosphamide; G, gintonin.

observed in IL-2 and IL-10 treatment, also IL-4 exhibited only a moderate stimulated response. Under normal conditions, the amount of serum expression of IL-4 is only minimal to maintain the basic activity of B cells. However, after CY injection the serum level of IL-4 in rats decreased to almost at the lower limit of detection, which resulted in abnormal B cell differentiation or activation. But most importantly, the serum level returned to normal level in rats treated with gintonin, indicating that gintonin could antagonize the immunosuppressive effect of cyclophosphamide on B cells (Fig. 3C). Western blot studies illustrates that gintonin treatment increased the expression levels of perforin, CD16, NKp30, and NKp44. Compared to CY group, the treatment with CY + G100 had 0.82 ± 0.10, 0.90 ± 0.11, 1.03 ± 0.38 and 0.53 ± 0.04, and for CY + G300 had 1.05 ± 0.13, 1.07 ± 0.04, 1.26 ± 0.03 and 0.67 ± 0.10 in perforin, CD16, NKp30 and NKp44 expressions respectively (Fig. 4A–D). Collectively it indicates that gintonin was effective in activating NK cells through the production of cytokines, and expression of NK cytolytic molecules as well as increased population of NK cells. The numbers of WBC, RBC, and lymphocyte, and the concentration of HGB and HCT were significantly decreased compared to normal control after 14 days in any of the groups, whereas, the number of WBC, RBC, and lymphocytes were increased in 100 and 300 mg/kg gintonin-treated groups than that observed in the CY-treated group (*$P < 0.05$ and **$P < 0.01$) Table 1. However, no statistically significant differences were found between groups for the changes of an anemia parameters such as mean corpuscular volume, and mean corpuscular hemoglobin concentration (data not shown). Regardless, flow cytometry analysis showed that NK markers such as NKG2D, NKp30, and NKp46 were upregulated, although granzyme B displayed only marginal increase compared to CY group. In addition, an increase in the expression of perforin, CD16, NKp30 and NKp44 were observed in western blot analysis. Based on these findings, it can be concluded that gintonin might play an immunomodulatory role to amplify the immune activity through NK cell activation and in our future studies we will evalulate the precise mechanism involved in immune-enhancing effect of gintonin.

Declaration of competing interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

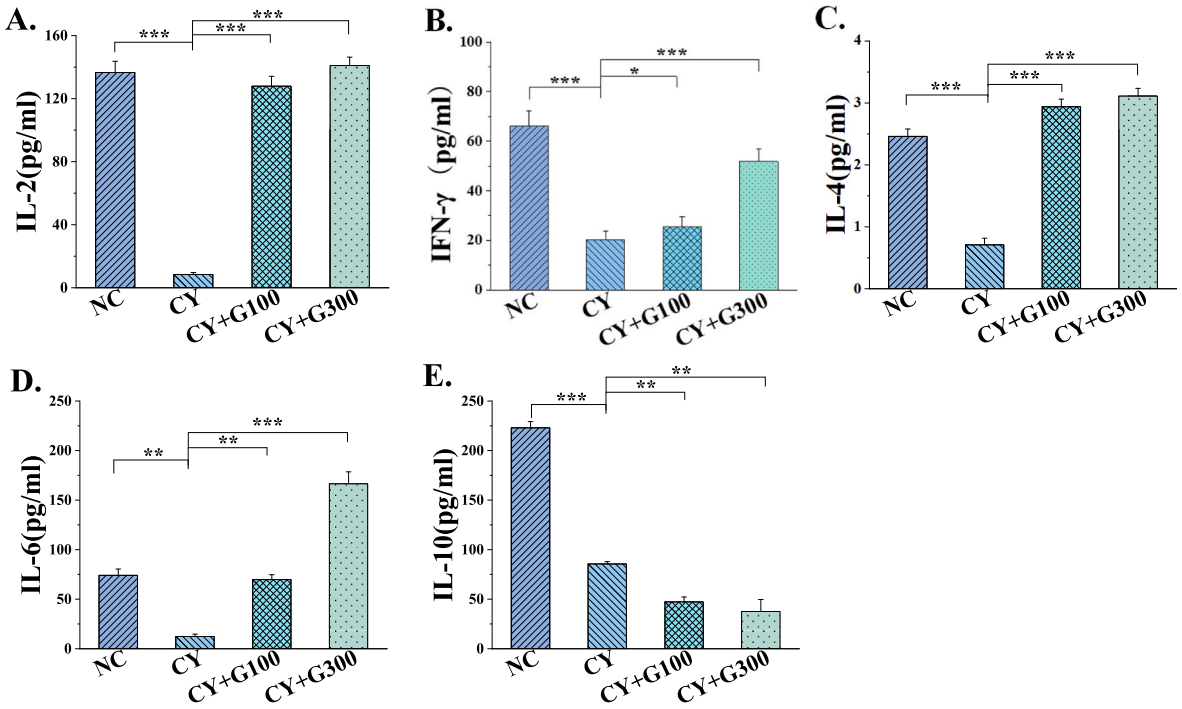

Fig. 3. ELISA analysis to quantitatively detect cytokine levels in each group. It was shown that the treatment of gintonin significantly increased the levels of cytokines such as IL-2 (A), IFN-r (B), IL-4 (C), and IL-6 (D), while the levels of IL-10 (an immunosuppressive cytokine) was reduced by the treatment of gintonin (E). All data are expressed as the means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the CY group. Abbreviation: NC, normal control; CY, cyclophosphamide G, gintonin.

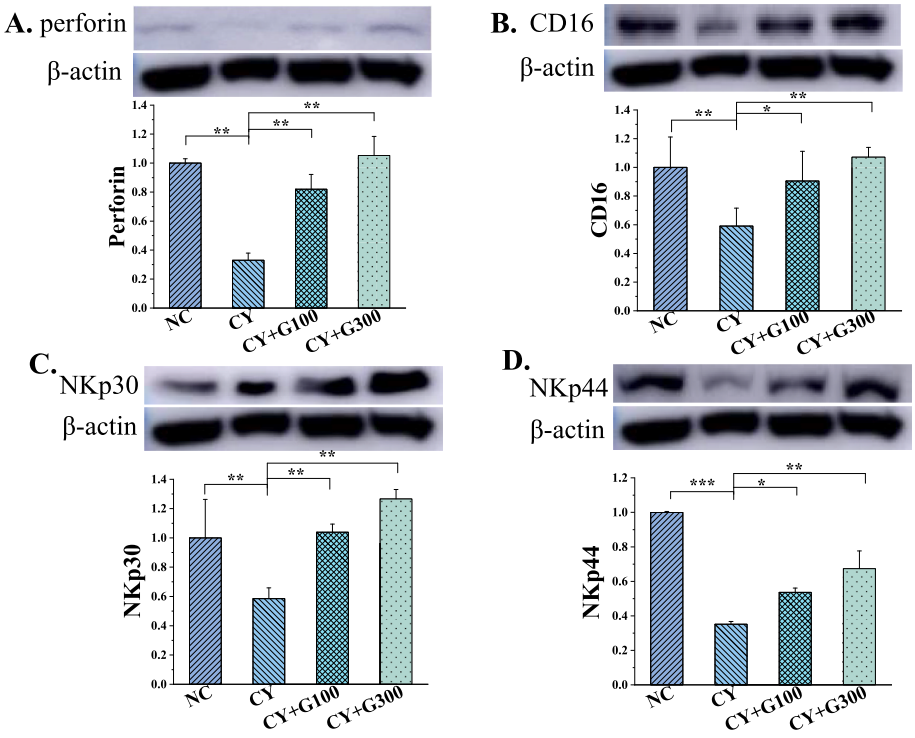

Fig. 4. Western blot analysis to detect the relative expression of perforin, CD16, NKP30, and NKP44 molecules. The levels of perforin, CD16, NKP30, and NKP44 proteins were increased by the treatment of gintonin with dose-dependent manner in each group. The β-actin as an internal reference for this experiment. All data are expressed as the means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the CY group. Abbreviation: NC, normal control; CY, cyclophosphamide G, gintonin.

Table 1
Results are expressed as the mean ± SE in each group.

Group	WBC (10^3 cells/μL)	RBC (10^6 cells/μL)	HGB (g/dL)	HCT%	Lymphocyte (10^3 cells/μL)
N/C	7.2 ± 0.32	7.75 ± 0.14	16.5 ± 0.46	44.83 ± 1.41	5.55 ± 0.25
CY	0.85 ± 0.53***	6.04 ± 0.34**	12.1 ± 0.84**	33.4 ± 1.33***	0.6 ± 0.56***
CY+100G	4.98 ± 1.28	7.61 ± 0.07	16.83 ± 0.12	45.37 ± 0.45	3.05 ± 0.67*
CY+300G	3.78 ± 1.33*	7.84 ± 0.33	17.18 ± 0.27*	46.75 ± 1.08	2.12 ± 0.47**

*Significantly different ($P < 0.05$) from N/C.
**Significantly different ($P < 0.01$) from N/C.
***Significantly different ($P < 0.001$) from N/C.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2023.12.001.

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