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FERTILITY STUDY (SEGMENT I) OF RECOMBINANT GRANULOCYTE-MACROPHAGE COLONY STIMULATING FACTORS (LBD-005) IN RATS

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ABSTRACT: *The recombinant glycoprotein, LBD-005 (Lucky R & D Center, Biotechnology) stimulates the growth of stem cells and activates the development of the hematopoietic cell in a similar manner to the naturally occurring GM-CSF. This test was conducted to investigate if LBD-005 had any effect on fertility in male and female rats. Sprague-Dawley rats (88 of each sex) bred at KRICT, were dosed subcutaneously, at a volume of 2 ml/kg body weight with LBD-005 at 0, 250, 500 or 1,000 μg/kg body weight. The male rats were dosed, from 6 weeks of age, for 60 days prior to mating. The females were dosed from 13 weeks of age for at least 14 day prior to mating and through to the 7th day of gestation. All the animals were killed by ether anesthesia followed by examination. The males were killed after successful mating, the females were killed on day 20 of gestation. No drug related effects were seen in any of the parameters measured : Water and food consumption, male and female body and organ weights, fertility index, number of corporus lutea, implantation and resorption sites, number of live fetuses, skeletal and visceral findings in fetuses. Therefore the no observed effect dose levels of LBD-005 are 1,000 μg/kg/day both for general toxicity and reproductive function in parent animals and for fetuses, which is 200 times the expected clinical dose.*

Key words: *Recombinant Granulocyte-Macrophage Colony Stimulating Factors (LBD-005), fertility study, Sprague-Dawley rat.*

INTRODUCTION

Granulocyte Macrophage Colony Stimulating Factors (LBD-005) (GM-CSF)

have been shown not only to stimulate the proliferation of granulocyte and macrophage colonies (Review Burgess & Metcalf, 1980; Sieff *et al.*, 1985; Mecalf *et al.*, 1986) but also to regulate some of the functional activities of stem cells (Review Metcalf, 1986). This has stimulated a great deal of research into the possible relationships of GM-CSF in combating myeloid leukemias and other leucocyte deficiency diseases (Gasson, 1984; Barlogie *et al.*, 1990).

It is very difficult to extract and purify large enough volumes of LBD-005 as a natural product for commercial purposes. This problem has been solved by Lucky R & D Center, Biotechnology (84, Jang Dong, Yousung Koo, Taejon, Korea) through the development of a recombinant form of GM-CSF (LBD-005).

This study was conducted to investigate the effect of LBD-005 on the reproductive capability by subcutaneous application through before mating to early gestation in rats. It was carried out in according to the "Guidelines for Reproduction Experiments to Evaluate the Safety of Drug" issued by the Ministry of Health, Korean Government in October 1988 (Segment I).

MATERIALS AND METHODS

Animals

Eighty eight male and 88 female, 4 week old, SPF Sprague-Dawley rats were supplied by the KRICT, Toxicology Center Breeding Facility, the animals were acclimatized for 10 days prior to the start of the study.

During the study the animals were maintained a strict SPF barrier, housed 5 animals cage. The cages were 260×420×180 mm polycarbonate with solid floors and wood chips (Sei Chang Co.) supplied as bedding. The bedding was changed weekly. The room was illuminated by fluorescent tubes at 200-300 lux and a 12 hour light/dark cycle (07 : 00-19 : 00). The temperature was maintained at 23± 3 °C, with a relative hummidity of 50± 10% and 13-18 air changes per hour.

Water, sterilized by UV light and standard laboratory rodent diet (Jeil Feed Co. Taejon, Korea) sterilized by irradiation at 2.0 Mrad was available *ad libitum*.

The rats were removed from the group housed cages and placed in pairs (1 ♂ and 1 ♀ /cage) just prior to mating and the females were individually caged during the gestation period.

Grouping and Identification

Prior to the start of the study all the animals were weighted and placed in body weight bands of 5 g, the animals were then selected into the 4 study groups (22 animal/sex/group) by the use of a computer generated random order. The animals were then individually identified by ear punching.

Experimental Design

Four groups of 22 male and 22 female rats were dosed subcutaneously at 0 (vehicle control), 250, 500 and 1,000 μg/kg body weight. Subcutaneous injection is the prefered clinical route and 1,000 μg/kg is 200 times the expected clinical dose.

The male rats were dosed daily from 6 weeks of age for 60 days until pairing

and then until successful mating occurred. The females were dosed from 13 weeks of age, daily for 14 days prior to pairing and then until day 7 of gestation.

Compound

LBD-005 was supplied by Lucky R & D Center, Biotechnology (84 Jang Dong, Yousung Koo, Taejon, Korea) with a protein content of 1 mg/1 ml w/v, pH 7.4 and an osmotic pressure, 283 mOsm.

The vehicle, phosphate buffered saline (pH 7.4) was used as the control solution. Dilutions were made up weekly according to the most recent body weight and all solutions were stored at 4°C and administered at room temperature.

Observations

The animals were examined twice daily for adverse clinical signs throughout the dosing period and daily during other stages of the study.

In all animals body weights were recorded weekly prior to mating. In addition, the females were weighed on day 0, 7, 14 and 20 of gestation.

Food consumption was estimated over the 24 hour period after the animals were weighted, except at the end of gestation when it was recorded from day 19.

Proof of mating was determined by copulation plug or when sperms were seen during the microscopical examination of the vaginal smears.

Necropsy

All animals were killed by an overdose of CO_2 anaesthesia followed by exsanguination and a full necropsy.

The following organs were weighted : liver, kidneys, spleen, heart, adrenals, brain, ovaries and testes. No tissues were taken for histopathological examination.

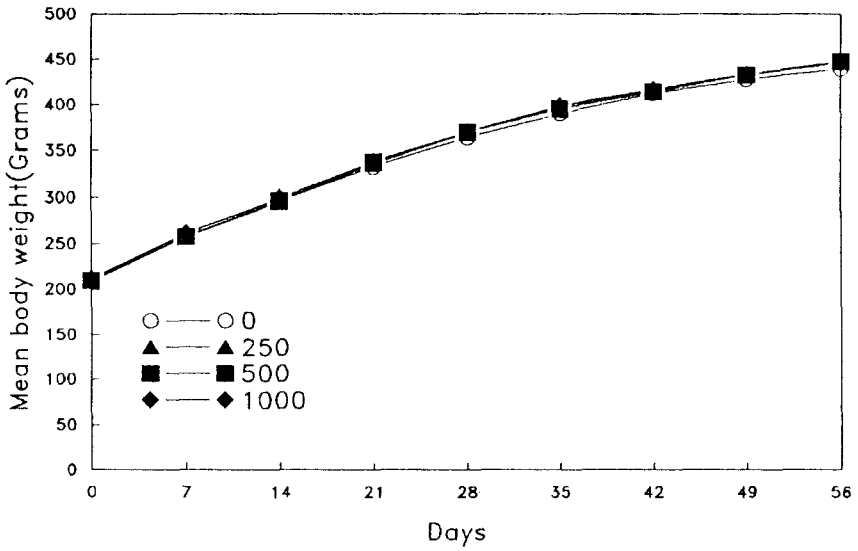

Figure 1. Changes in mean body weight of male rat treated subcutaneously with LBD-005 during the pre-mating period.

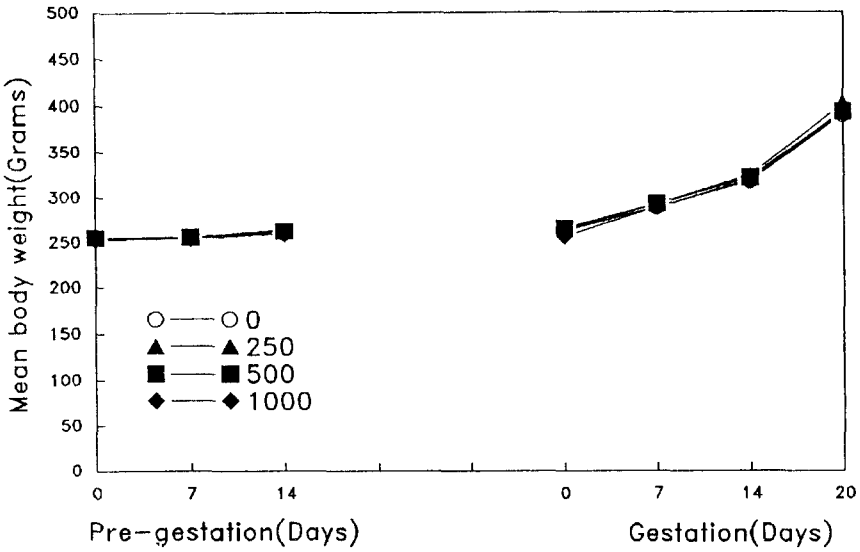

Figure 2. Changes in mean body weight of female rat treated subcutaneously with LBD-005 during pre-mating and early gestation period.

In addition, pregnant females had the following checked and the numbers recorded : corporus leutea, implantation sites, resorption sites, live and dead fetuses. The individual body weights and sex of live fetuses were recorded.

Examination of Fetuses

The position of each fetuses in the uterus was recorded and alternate fetuses were selected for either skeletal examination by the Inouye (1976) Method or visceral examination by the Wilson's Method (Wilson and Warkany, 1972) for the head and abdomen and Nishimura (1974) Method for the thorax.

Statistical Analysis

All data collected were subjected to analysis by Dunnett and Kruskal-Wallis test.

RESULTS

General Effects of the Administration of LBD-005 in Male Rats

The treated animals did not show any abnorml response to the treatment and were reanimated in good physical conditions as the control animals were. No death occurred during the experiment period. As indicated in Fig. 1, the body weight changes of treated and control groups were not significantly different. Food consumptions were similar in all groups as shown in Fig. 3. At necropsy of the males after pairing, no pathological changes related to the treatment detected.

In relative organ weights, no significant difference was found as compared with that of the control group (Table 1).

General Effects of the Administration of LBD-005 in Female Rats

Table 1. Effect of subcutaneous injection of LBD-005 on the relative organ weights of F0 male rats

Dosage (μg/kg)	0	250	500	1000
Body weight (G)	466.4	470.7	475.8	471.2
SD	34.06	37.69	45.65	33.99
Number of animals	22	22	22	22
Liver (G)				
% Body weight	4.612	4.643	4.361	4.291
SD	0.4891	0.5683	0.6066	0.5121
Kidney-left (G)>				
% Body weight	0.404	0.398	0.393	0.394
SD	0.0290	0.0361	0.0362	0.0444
Kidney-right (G)>				
% Body weight	0.415	0.405	0.404	0.406
SD	0.0276	0.0354	0.0347	0.0506
Spleen (G)				
% Body weight	0.161	0.174	0.168	0.171
SD	0.0195	0.0220	0.0222	0.0169
Heart (G)				
% Body weight	0.321	0.356	0.340	0.335
SD	0.0280	0.0452	0.0360	0.0297
Brain (G)				
% Body weight	0.461	0.443	0.446	0.447
SD	0.0322	0.0327	0.0432	0.0420
Adrenal gland-left (G)>				
% Body weight	0.006	0.007	0.007	0.006
SD	0.0010	0.0014	0.0013	0.0012
Adrenal gland-right (G)>				
% Body weight	0.006	0.006	0.006	0.006
SD	0.0009	0.0010	0.0010	0.0010
Testis-left (G)>				
% Body weight	0.344	0.358	0.347	0.370
SD	0.0634	0.0359	0.0670	0.0611
Testis-right (G)>				
% Body weight	0.343	0.362	0.350	0.359
SD	0.0638	0.0368	0.0700	0.0301

Analysis of Variance using DUNNETT'S Procedure
* Significantly different from control value at $p<0.05$.
**Significantly different from control value at $p<0.01$.

No death occurred in both the control and treated groups. The treated females were in good condition as those of the control group during the experimental period. Before pairing, and during gestation mean body weight of the treated groups was similar to that of the control as shown in Fig. 2. Food consumption

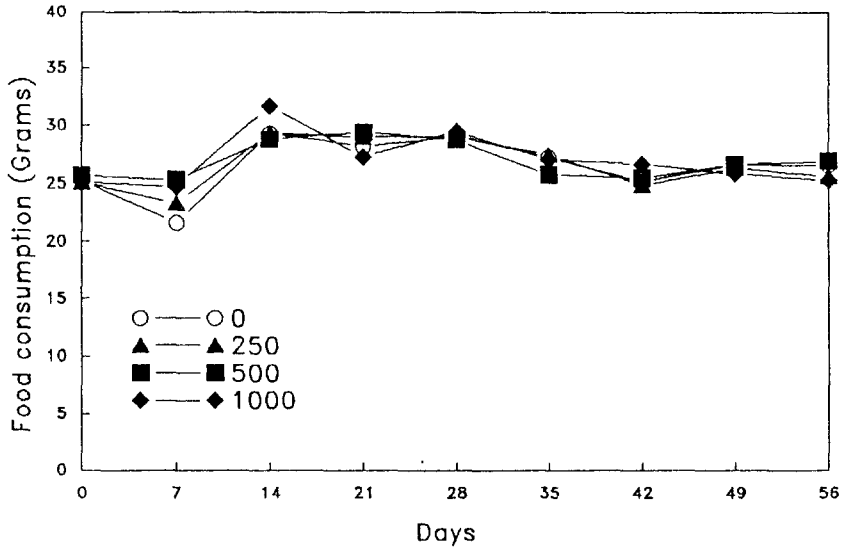

Figure 3. Changes in food consumption of male rat treated subcutaneously with LBD-005 during the pre-mating period.

was similar in all groups before pairing and during gestation (Fig. 4).

At necropsy of all dams after the cesarean section, no evidences of any treatment-related change were apparent.

Effects of the Administration of LBD-005 on Parental Fertility

Copulation rate was 95.5% in the control 500 μg/kg and 1,000 μg/kg and 100% in 250 μg/kg. The ratios of fertility (male) and pregnancy (female) in the control, 250, 500 and 1,000 μg/kg group were 81%, 95.5%, 90.5% and 90.5%, respectively (Table 3).

Effects of the Administration of LBD-005 in Fetuses

As shown in Table 4, examination on fetuses delivered by the cesarean section at day 20 of gestation revealed no dose-related changes attributable to the treatment with LBD-005 in the number of implantations and resorptions, in the sex ratio of fetuses and in fetal body weight.

No drug related effects were seen in skeletal and visceral examination of the fetuses (Table 5, 6).

DISCUSSION

A fertility study of LBD-005 was carried out in SD rats subcutaneously injected at dose levels of 250, 500 and 1,000 μg/kg. These dosages correspond to 50, 100 and 200 times of the provided clinical use level of 5 μg/kg of body weight. Male rats were treated from 60 days before pairing until the completion of mating. Female rats were injected LBD-005 from 14 days prior to mating up to day 7 of gestation and all fetuses were examined for abnormalities.

Table 2. Effect of subcutaneous injection of LBD-005 on the relative organ weights of F0 female rats

Dosage (μg/kg)	0	250	500	1000
Body weight (G)	390.7	402.2	393.9	392.5
SD	26.40	24.89	28.86	28.63
Number of animals	17	21	18	19
Dunnett's				
Liver (G)				
% Body weight	4.139	4.198	4.066	4.173
SD	0.3067	0.2886	0.2992	0.5105
Kidney-left (G)>				
% Body weight	0.288	0.284	0.281	0.285
SD	0.0278	0.0255	0.0252	0.0330
Kidney-right (G)>				
% Body weight	0.299	0.292	0.287	0.292
SD	0.0243	0.0257	0.0231	0.0344
Spleen (G)				
% Body weight	0.164	0.164	0.163	0.171
SD	0.0153	0.0187	0.0132	0.0211
Heart (G)				
% Body weight	0.247	0.241	0.244	0.243
SD	0.0210	0.0201	0.0236	0.0233
Brain (G)				
% Body weight	0.503	0.490	0.503	0.505
SD	0.0354	0.0327	0.0306	0.0446
Adrenal gland-left (G)>				
% Body weight	0.009	0.010	0.009	0.010
SD	0.0017	0.0017	0.0012	0.0016
Adrenal gland-right (G)>				
% Body weight	0.009	0.009	0.009	0.009
SD	0.0013	0.0016	0.0013	0.0016
Overy-left (G)>				
% Body weight	0.014	0.015	0.014	0.016
SD	0.0035	0.0024	0.0022	0.0038
Overy-right (G)>				
% Body weight	0.015	0.015	0.014	0.016
SD	0.0020	0.0028	0.0023	0.0027

Analysis of Variance using Dunnett's Procedure
* Significantly different from control value at $p<0.05$.
**Significantly different from control value at $p<0.01$.

Food consumption was not affected by LBD-005, and no death was observed during the experimental period. No treatment-related change in body weight was also seen in both the control group and the treated groups. Male and Female rats used in the experiment showed no special alterations or toxic lesions with the

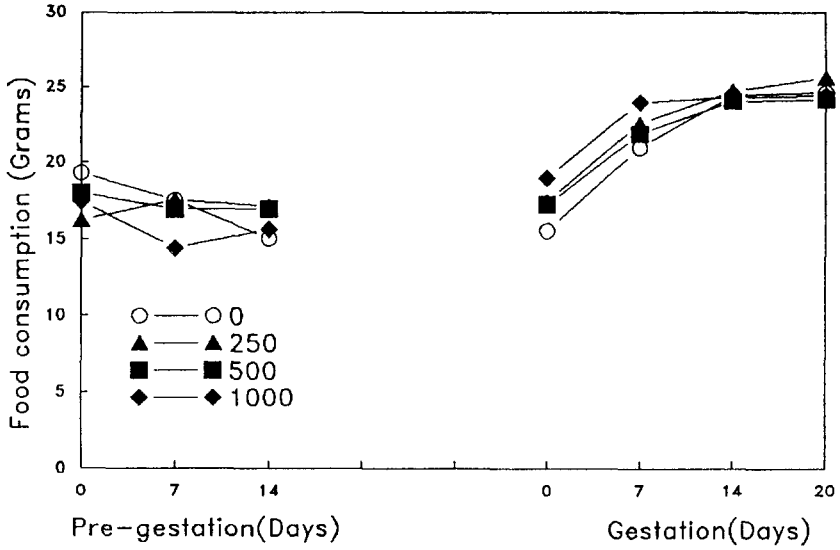

Figure 4. Changes in food consumption of female rat treated subcutaneously with LBD-005 during the pre-mating and gestation period.

Table 3. Effects on fertility of parent animals treated subcutaneously with LBD-005

Dose (μg/kg)	0	250	500	1000
No. of mated animal				
Male	22	22	22	22
Female	22	22	22	22
No. of copulated animal				
Male	21	22	21	21
Female	21	22	21	21
No. of impregnated male	17	21	19	19
No. of pregnant female	17	21	19[a]	19
Male				
Copulation index[b]	95.5	100.0	95.5	95.5
Fertility index[c]	81.0	95.5	90.5	90.5
Female				
Copulation index[b]	95.5	100.0	95.5	95.5
Prognancy index[d]	81.0	95.5	90.5	90.5

[a] Neither vaginal plug nor sperm was detected in one animal but the delivered fetuses.
[b] (Total No. of copulated animals)/(No. of mated animals)×100
[c] (Total No. of impregnated animals)/(No. of animals with successful copulation animals)×100
[d] (Total No. of pregnant animals)/(No. of animals with successful copulation)×100.

exception of the significant increase of the heart weight in 250 μg/kg group of males and the ovary weight in 250 μg/kg and 1,000 μg/kg group of females. However, no dose-response was detected. No abnormal signs were seen in mating or fertility in rats injected with LBD-005.

Table 4. Effects on findings at caesarean section of dams treated with LBD-005

Dose (μg/kg)	0	250	500	1000
No. of pregnant animals	17	21	18	19
Corpora lutae				
Mean± S.D.	15.29± 2.59	16.48± 3.23	15.83± 3.17	16.47± 1.81
Implantations				
Mean± S.D.	13.71± 2.62	14.10± 3.08	13.94± 2.90	15.00± 1.29
% to corpora lutea : Mean± S.D.	89.52± 7.01	85.43± 7.23	88.14± 11.56	91.39± 1.29
Fetal death (resorption + dead fetuses)	12	12	6	16
Resorption				
Total	10	12	6	14
Early	10	11	6	13
Late	0	1	0	1
Dead fetuses	2	0	0	2
Live fetuses				
Male/Female[a]	93/127	127/157	127/118	132/137
Mean± S.D.	12.94± 2.22	13.52± 2.84	13.61± 2.97	14.16± 1.64
% to implantation : Mean± S.D.	95.13± 7.54	96.47± 7.19	97.55± 3.57	94.68± 7.51
Sex ratio (male/female)	0.73	0.81	1.08	0.96
External anomalies of fetuses	0	0	0	0
Body weight of alive fetuses				
Male : Mean± S.D.	3.48± 0.27	3.65± 0.51	3.65± 0.78	3.51± 0.26
Female : Mean± S.D.	3.32± 0.22	3.54± 0.61	3.50± 0.84	3.35± 0.23

Kruskal-Wallis using Scheffe's Procedure
[a] X^2 test.

Table 5. Visceral findings in F1 fetuses from F0 dams treated subcutaneously with LBD-005 during the pre-mating and early gestation period

Dose (μg/kg)	0	250	500	1000
No. of dams	17	21	18	19
No. of live fetuses tested	105 (6.18± 1.13)[a]	136 (6.48± 1.50)	118 (6.56± 1.50)	131 (6.89± 0.94)
No. of fetuses with malformation	0	2	0	0
No. of fetuses with variation	5	5	2	4
Head				
Palate	0	0	0	0
Ear	0	0	0	0
Lateral ventricle	0	0	0	0
Eye	0	0	0	0
Nasal cavity	0	0	0	0

Dose (μg/kg)	0	250	500	1000
Thorax				
Diaphragm	0	0	0	0
Irregular shape of left thymus	1	1	0	0
Lung	0	0	0	0
Vessels	0	0	0	0
Interventricular septal defect	0	1	0	0
Abdomen				
Triple kidney (left)	0	1	0	0
Dilatation of renal pelvis	1	1	0	2
Dilatation of ureter	3	3	2	2
Gonad	0	0	0	0

Kruskal-Wallis using Scheffe's Procedure
[a] Each values represent total (Mean± S.D.).

Table 6. Skeletal findings in F1 fetuses from F0 dams treated* subcutaneously with LBD-005 during the pre-mating and early gestation period

Dose (μg/kg)	0	250	500	1000
No. of dams	17	21	18	19
No. of live fetuses tested	115 (6.8± 1.15)	140 (7.0± 1.41)	127 (7.1± 1.51)	139 (7.3± 0.89)
Abnormality				
Skull	0	0	0	0
Sternum	0	1[a]	0	0
Vertebra	0	0	0	0
Ribs	0	0	0	0
Coxa	0	0	0	0
Fore limb	0	0	0	0
Hind limb	0	0	0	0
Variation				
No. of pre-sacral vertebrae				
25	0	0	0	0
26(standard)	115	140	127	139
27	0	0	0	0
No. of ribs				
12	0	0	0	0
13(standard)	115	139	127	138
14	0	1	0	1
Asymmetric sternebrae	1	0	0	0
Cervical rib	0	0	0	0
Thoracic and lumbar vertebral body	0	0	0	0
Ossification				
No. of fetuses with fully ossified squama occipitalis	0	0	0	0
No. of ossified sternebrae	4.4± 0.57	4.6± 0.64	4.5± 0.86	4.2± 0.88

Dose (μg/kg)	0	250	500	1000
No. of ossified metacarpals in both forelimbs	5.3± 1.59	5.8± 1.24	5.9± 1.53	5.3± 1.47
No. of ossified 1st phalanges in both forelimbs	0	0.4± 1.53	0.7± 2.12	0
No. of ossified metatarsals in both hindlimbs	5.6± 3.36	6.2± 3.09	6.7± 2.86	5.0± 3.87
No. of ossified 1st phalanges in both hindlimbs	0	0	0.4± 1.89	0
No. of sacral and caudal vertebrae	6.6± 1.34	6.9± 1.54	7.0± 2.00	6.0± 2.11

Kruskal-Wallis using Scheffe's Procedure

[a] Split of 4th sternebrae.

* Significantly different from control value at $p<0.05$.

**Significantly different from control value at $p<0.01$.

The occurrence of external, internal and skeletal anormalies in a few fetuses of all experimental groups, including the control group, might be considered to be as physiological variation (Manson and Kang, 1989; Morita *et al.*, 1987) of this strain of rats and is unrelated to the injection of the substance.

From the results mentioned above, it may be concluded that LBD-005 dose not appear to produce significant changes in mating, fertilization, implantation or embryonic development, even when injected subcutaneously at 1,000 μg/kg of body weight.

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