The thesis is about the effects of Traditional Chinese Medicine, Ekki-Youketsu-Fusei-Zai, on survival and NK cell function of tumor-bearing mice and on cytokine production by murine macrophage cell line J774.1.

**Abstract:** We examined anti-tumor effects of the Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), on survival, tumor size, body weight, and natural killer (NK) cell activity of tumor-bearing mice by using a tumor cell line, colon-26. A significant life-prolonging effect was found, when EYFZ was orally administrated for 28 days, which started just after the subcutaneous implantation of colon-26 cell line. In addition, an oral administration of EYFZ inhibited the tumor growth and the loss of body weight in tumor-bearing mice. The significant increases in splenic NK cell activity of the tumor-bearing mice were also induced by oral administration of EYFZ. To elucidate the mechanisms of these anti-tumor effects, we further investigated the effect of EYFZ on cytokine production by murine macrophage-like cell line, J774.1 cells originating from BALB/c mice. In this experiment, we examined 6 kinds of cytokine mRNA expression by J774.1 cell in response to EYFZ-stimulation. RT-PCR revealed that both interleukin-12 (IL-12)p35 and IL-12p40 mRNA expression were induced by EYFZ treatment. Moreover, EYFZ promoted the secretion of IL-12 from J774.1 cells. These results strongly suggest that EYFZ has the anti-tumor effects on colon-26 implanted mice via augmentation of NK cell activity in vivo and induce the functional activation of J774.1 cells in vitro.

**Keywords:** Traditional Chinese Medicine (TCM), Ekki-Youketsu-Fusei-Zai (EYFZ), anti-tumor activity, natural killer (NK) cell activity, murine macrophage cell line J774.1, IL-12 mRNA expression, IL-12 production.

**Introduction**

Some kinds of crude drugs of Traditional Chinese Medicine (TCM) have been so far useful for cancer patients as both an anti-tumor drug and an adjuvant, which greatly enhance the immunological functions of cancer patients. Ekki-Youketsu-Fusei-Zai (EYFZ), one of the TCMs, is a mixture composed of six kinds of crude drugs, which have been used for cancer patients in China. Recent studies have shown that each crude drug has various biological activities. For example, *Astragali radix* has immunologically enhancing and anti-tumor effects, while *Angelicae radix* has anti-inflammatory, analgesic, interferon inducing, and immunopotentiating effects. *Cervi parvum cornu* is known to show anti-tumor and immuno-enhancing effects by inhibiting the monoamine oxidase (MAO) activity, and *Zizyphi fructus* augments the function of natural killer cell as well as ciliary motility in the airway. *Rehmanniae radix* is reported to have anti-tumor activity, enhance cytotoxic T lymphocytes (CTL) activity and induce IL-2 production by T cells.
Macrophages are known to be activated in vitro by a variety of agents, leading to an increase of metabolism, syntheses and release of lysosomal enzyme, phagocytosis, and anti-mitotic or anti-tumor effects. Many of these agents are generally potent immunological adjuvants or immunopotentiators, some of which are natural products called biological response modifiers (BRM) and possess functions affecting immune system and host defense mechanisms. BRM also alters the host biological responses, and is effective in suppressing tumor cells. Among these products, bacterial lipopolysaccharides (LPS) is one of the strongest activators of macrophage. To verify the mechanisms underlying the LPS action on macrophage, murine macrophage-like cell line, J774.1 has been so far used in many investigations.

Though each component of EYFZ has shown various biological activities and EYFZ has been used for cancer patients, its anti-tumor effects and immuno-enhancing effects have not been investigated. In this study, we examined the effects of oral administration of EYFZ on the survival, tumor size, body weight and natural killer (NK) cell activity of tumor-bearing mice in vivo and also examined the effect of EYFZ on cytokine production of J774.1 in vitro in order to elucidate the relationship between immuno-enhancing effects of EYFZ and its anti-tumor effects.

Materials and Methods

Animals and tumor cell line Specific-pathogen-free BALB/c female mice at the age of 5 weeks, originally purchased from Japan CLEA Co, were used throughout this experiment and approved by institutional animal care committee. Colon-26 cell line, colon-adenocarcinoma from BALB/c mice, was generously provided by Prof. Kikuo Nomoto, Kyushu University, and cultured in vitro with the basal medium (RPMI1640 with 2.0 g/L NaHCO₃, 1.0 g/L HEPES, 0.6 g/L-glutamine, and 0.25 mg/L Kanamycin) containing 10% fetal bovine serum (FBS). By a strong pipetting procedure, we obtained a single cell suspension and 5 × 10⁵ cells were implanted subcutaneously into the back of mouse. Murine lymphoma YAC-1 cell line was maintained in the same culture medium as shown above.

Preparation of EYFZ EYFZ is a mixture composed of six kinds of crude drugs as shown in Table 1. All of the crude drugs were obtained from Tochimoto, Ltd., Osaka, and EYFZ was prepared as follows. First, a mixture of Astragali radix (10.0 g), Zizyphi fructus (5.0 g), Amomi semen (5.0 g), Angelica radix (8.0 g), Cervi parvum cornu (5.0 g), and Rehmanniae radix (10.0 g) was added to 200 ml distilled water (DW) and soaked for 20 min at room temperature. Then, it was boiled for 30 min for extraction of effective substances, and the solution was centrifuged at 3000 rpm for 20 min. Finally, the supernatant was condensed to 43 ml (1 g crude drugs/ml) by heating evaporation, and diluted with DW to desired concentrations for in vivo and in vitro experiments, and orally administered to mice at a dose of 716.7 mg crude drugs/kg. This dose was considered appropriate because oral administration of EYFZ into human is traditionally 43 g crude drugs/60 kg/day.

Evaluation of survival, tumor size, and body weight of tumor-bearing mice Colon-26 (5 × 10⁶) cells were subcutaneously implanted into 6-week-old mouse at once, and simultaneously the oral administration of EYFZ was started. Control mice received the same volume of saline instead of EYFZ. Thereafter, the survival of these mice was monitored everyday for evaluating the life-prolonging effect. The tumor size and body weight were examined 2-3 times a week. The major axis (a) and minor axis (b) of the tumor were measured, and then size was estimated by using the formula ab²/2³. Assay of splenic NK cell activity The splenic NK cell activity of tumor-bearing mice was determined by lactate dehydrogenase (LDH) assay. The spleen cell suspension was prepared by squeezing the spleen between two glass slides. The distilled water was added to the spleen cell suspension for provoking lysis of red blood cells. After washing three times with serum-free basal medium, the cells were incubated in a 25 cm² culture flask (FALCON, Becton Dickinson) in serum-free basal medium at 37 °C in a 5 % CO₂ incubator for 2 hr to remove adherent cells. The non-adherent cells were collected as effector cells. Effector cells (5 × 10⁶ cells/ml) were incubated with NK-sensitive target cells, YAC-1 (5 × 10⁵ cells/ml), in a total volume of 0.2 ml/well using 96 well round bottomed microplate (IWAKI Glass Co, Ltd.). An effector:target ratio of 100:1 was considered optimum. The plate was incubated for 4 hr at 37 °C in a 5 % CO₂ incubator. After incubation, 0.05 ml of the supernatant from each well was collected, then used for LDH assay to determine the cytotoxic activity using the LDH cytotoxic Kit (Wako Pure Chemical Industries, Ltd.). The percentage of specific release was calculated according to the following formula: % specific lytic activity = (experiment
release · spontaneous release) / (maximum release · spontaneous release) × 100

**J774.1 cell line culture** The murine macrophage-like cell line, J774.1, was obtained from Riken Cell Bank (RCB, Japan). The cells were maintained in 75 cm² plastic culture flasks (Falcon) in basal medium containing 5% fetal bovine serum. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells harvested by gentle scraping were passaged every 3-4 days by diluting 1:10 with fresh medium.

**RT-PCR** J774.1 cells were suspended in culture medium at a cell concentration of 5×10⁴ cells/ml, and 5 ml of the cell suspension was plated in a 25 cm² plastic culture flask (Falcon). Cells were cultured for 24 and 48 hrs in the presence of 1.2 mg crude drugs/ml of EYFZ, 10 ng/ml LPS or culture medium only, respectively, and after incubation, cells were harvested by scraping and quickly frozen and stored at -80°C until use.

**Statistical analysis** Survival curve was determined using the method of Kaplan and Meier, and the log rank test was used to calculate the significance. Other data were statistically analyzed based on the Student's t test, and the differences were recognized significant with p value less than 0.05. The results were expressed as mean ± standard deviation (SD).

**Results**

**Effect of EYFZ on a life-prolongation, tumor size and the body weight of tumor bearing mice**

We first examined the effect of oral administration of EYFZ on the survival of tumor-bearing mice. When EYFZ (716.7 mg crude drugs/kg/day) was continuously administrated to the mice which had been implanted subcutaneously with colon-26 for 28 days, the

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### Table 1. The Botanical Origins of Crude Drugs of "Ekki-Youketsu-Fusei-Zai"

<table>
<thead>
<tr>
<th>Crude drug</th>
<th>Botanical origin (Family name)</th>
<th>Harvesting time</th>
<th>Representative defined compounds</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Astragalus Radix</td>
<td>* A. membranaceus (R. Br.) Var. rugosus (R. Br.), A. mongholicus (R. Br.)</td>
<td>spring or autumn</td>
<td>β-sitosterol, D-β-Angelone, Diosgenin, 2,4-dihydroxy-5,6-dimethoxy-flavanone, calystegine, furostemonol, astrogastrolide 1, I, IV, V</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Ziziphus Fructus</td>
<td>* Z. jujuba (Rhamnaceae)</td>
<td>autumn</td>
<td>Sugar, phlegm, malic acid, tartaric acid</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Anemone Semen</td>
<td>* A. eulamnorum.</td>
<td>autumn</td>
<td>Bornesol, deoxyrhamycin, bornyl acetate, bornylic acid</td>
<td>5.0 g</td>
</tr>
<tr>
<td></td>
<td>* A. eulamnorum Wall.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angelicae Radix</td>
<td>* A. angustifolia (Oliv. &amp; Diels)</td>
<td>autumn</td>
<td>Lipotolid, n-butylenediphthalide, sesquilactone AR, curvared</td>
<td>5.0 g</td>
</tr>
<tr>
<td>Cervus Parvum Cornu</td>
<td>* C. nippon Temminck</td>
<td>spring or autumn</td>
<td>Ceramide, estosterone, 17-β-estradiol</td>
<td>5.0 g</td>
</tr>
<tr>
<td></td>
<td>* C. elaphus L.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rehmanniae Radix</td>
<td>* R. glutinosa L.</td>
<td>autumn</td>
<td>Catalpol, stachyose, amino acid, β-sitosterol</td>
<td>10.0 g</td>
</tr>
</tbody>
</table>

*Medicine was prepared by blending the crude drugs in the ratios indicated above.*
life-prolonging effect was found as shown in Fig. 1. All of tumor-bearing mice in the control group that received only the saline died within 59 days after the onset of this experiment. On the other hand, the tumor-bearing mice treated with EYFZ showed a significant life-prolonging effect as compared to the control (p < 0.01), and died in 70 days on average.

Second, we compared the tumor size of EYFZ-administrated group mice with that of control group mice. The tumor size, as described in Materials and Methods, was calculated by measuring the major (a) and minor (b) axis of formed tumor tissue based on the formula \( ab^{\frac{1}{2}} \). Result obtained from observation for 34 days revealed that the tumor size in EYFZ-administrated mice was smaller than that in the control mice (Fig. 2). Although the tumor size on day-13 was almost similar to that in the control, those on day-20 and on day-27 were clearly smaller than that in the control group (p < 0.5 and p < 0.1, respectively).

Third, we examined the effect of oral administration of EYFZ on the body weight in tumor-bearing mice. When we observed the body weight of mice in the EYFZ-treated or control mice successively for 28 days after the onset of this experiment, it was shown that the body weight in the EYFZ-treated mice was much larger than that in the control mice (Fig. 3). Particularly, the body weight in the treated mice was significantly larger than that in the control mice on day-14 (p < 0.05).

**Effect of EYFZ on NK cell activity of tumor-bearing mice** We examined the effect of oral administration of EYFZ on splenic NK cell cytotoxicity in tumor-bearing mice. We found that the NK cytotoxic activity of splenic cells in orally EYFZ-administrated mice was significantly higher than that in control mice on the day-17 and day-24 after the onset of transplantation and oral administration, as shown in Fig. 4 (p < 0.01).

**Effect of EYFZ on the expression of cytokine mRNAs in J774.1 cells** The cytokine mRNA expression of J774.1 cells treated with or without EYFZ was investigated. The gel electrophoretic patterns of the RT-PCR products of IL-1β, IL-12p35, IL-12p40, IFN-γ, TNF-α and β-actin are presented in Fig. 5. The expression level of IL-12p35 and IL-12p40 was induced in J774.1 cells after 12hrs and 24hrs of the treatment.

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Fig. 1. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on survival of mice inoculated with colon-26 cells. Six BALB/c female mice per group were inoculated s.c. with colon-26 (5x10⁵ cells/mouse). Treatment group mice were orally administered with EYFZ (716.7mg crude drugs/kg) for 28 days just after the subcutaneous implantation of colon-26 cell line. The control group mice received only saline. Their survival rates are shown. (●): treatment group mice, (○): control group mice. P < 0.01; by log rank test.

Fig. 2. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on the in vivo growth of colon-26 tumor cells. Mice treated with EYFZ (716.7mg crude drugs/kg) and control mice without EYFZ were inoculated with colon-26 cells according to the same protocol as in Fig. 1. Tumor growth was measured after implantation and calculated as shown in Material and Methods. The tumor size was measured from day-9 to day-34 after transplantation. (●): treatment group mice, (○): control group mice.

Fig. 3. Effect of Ekki-Youketsu-Fusei-Zai (EYFZ) on body weight loss of mice inoculated with colon-26 cells. Mice treated with EYFZ (716.7mg crude drugs/kg) and control mice without EYFZ were inoculated with colon-26 cells according to the same protocol as in Fig. 1. After implantation, the body weight was measured 2 or 3 times a week and finally on day-28. (●): treatment group mice, (○): control group mice.
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with EYFZ, though the expression of β-actin, a housekeeping gene, was almost constant in each sample. We could not find any differences of other cytokine expressions between treated and untreated cells.

**Effect of EYFZ on cytokine production in J774.1 cells** We next examined whether or not this increased expression of IL-12 induced by EYFZ occurs at the protein level. After J774.1 cells were incubated with 0.15 mg/ml, 1.2 mg/ml and 3.6 mg crude drugs/ml of EYFZ for 24, 48 and 72 hrs, IL-12 in the culture supernatants was assayed by ELISA. As shown in Fig. 6, IL-12 secreted from J774.1 cells stimulated with EYFZ in the culture supernatants was significantly higher in concentration compared to those of the control.

**Discussion**

In this experiment, we examined whether or not EYFZ, one of the TCMs, affected the anti-tumor activity in mice into which the murine colon-26 carcinoma cell line was subcutaneously implanted. This colon-26 cell line, an undifferentiated carcinoma induced by the carcinogen N-nitroso-N-methylurethan, has been successfully used as the model of tumor-bearing mice and cachexia.\(^{31,34}\) In our study, colon-26 cells could grow well after subcutaneous implantation into the normal BALB/c mice. Oral administration of EYFZ caused a statistically significant prolonging effect on survival in tumor-bearing mice as compared with the control mice. We also examined both the tumor size and body weight. The tumor size in EYFZ-administrated mice was shown to be much more decreased than that in the control mice. Particularly, the tumor size in the treated mice after 27 days became significantly smaller than that in the control. Hence, it is suggested that the oral administration of EYFZ was more effective compared with oral administration of saline for both the survival and the decrease of a tumor size in tumor-bearing mice.

The cachexia, an exhaustive state with severe weight loss, is a serious problem in cancer patients affecting their morbidity and mortality. It lowers their quality of life and shortens their life-span.\(^{35,36}\) Colon-26 cell line has been successfully used as the model of such cachexia by tumor growth.\(^{31}\) Thus, we observed the body weight of tumor-bearing mice everyday following the subcutaneous implantation of colon-26 cells into both the EYFZ-treated and the control mice. Results clearly showed that oral administration of EYFZ led to the better increase of body weight as compared to the control without EYFZ. Though the body weight of the control mice was initially lower than that of...
the EYFZ-treated mice, it finally reached the similar level to that of the EYFZ-treated mice. This means the tumor growth in the control mice leading to the increase of body weight. We think the difference of the body weight between the two groups of mice on day-14 is much more important than that on the later days, and it reflects the improvement of cachexia by EYFZ. However, since there was a possibility that EYFZ influenced the increase of the body weight of cancer-bearing mice from the nutritious viewpoints, it will be necessary to further examine the effect of oral-administration of EYFZ on body weight of the normal mice without cancer cell in the future.

NK cells exhibit spontaneous cytotoxic activity in a non-major histocompatibility complex (MHC) restricted manner against virus-infected cells and cancer cells in vivo and their activity can be augmented by administration of interferon-γ (IFN-γ) \textsuperscript{35-38}. Some papers showed that some kinds of crude drugs and TCMs exert anti-tumor effects by activation of NK cells \textsuperscript{13,39-43}. Therefore, we also tried to examine the cytotoxic effect of oral administration of EYFZ on splenic NK cell activity in tumor-bearing mice on day-10, day-17, day-24 and day-31 following the subcutaneous implantation of colon-26 cells. Results clearly showed that the NK activity of spleen cells in orally EYFZ-administrated mice was significantly higher than that in control mice on the day-17 and day-24 after transplantation and oral EYFZ administration. In our present experiment, it was hard to investigate the cytokine production of NK cells in the spleen due to the insufficient number of splenic NK cells. However, as the NK activity was clearly augmented in the EYFZ-treated mice, further evaluation for EYFZ in anti-tumor activity seems to be needed by examining the effect of oral-administration of EYFZ on the cytokine production of NK cells in the spleen.

EYFZ does not show the direct cytotoxicity on some tumor cell lines including colon-26, A549 and Kato III (data not shown). This greatly suggests that the efficacy of EYFZ on survival and tumor-growth in tumor-bearing mice is attributable to the enhancement of host defense or immune system such as NK cell activity but not to the direct cytotoxicity against tumor cells.

To elucidate the relationship between the macrophage and anti-tumor effects of EYFZ, we also tried to examine the effects of oral administration of EYFZ on the functions of peritoneal macrophage in tumor-bearing BALB/c mice; however, it was difficult to get the some effects of EYFZ on macrophage functions. First, for example, mice got weak after the implantation of colon-26 cell, and it was difficult to collect enough cells of resting macrophage without thioglycollate-stimulation. Second, since the thioglycollate itself strongly stimulated the resting peritoneal macrophage in tumor-bearing mice, we could not find any difference between the EYFZ-administrated and control mice on functions of peritoneal macrophage. Third, J774.1 macrophage-like cell line was originated from the BALB/c mice and can be used for the study of macrophage function in vitro because this cell line...
was reported to show the same cytokine production as the macrophages\textsuperscript{44-47}. Thus, in the present study, we examined the effect of EYFZ on cytokine production of J774.1 \textit{in vitro} to elucidate the mechanisms underlying the EYFZ action on immune responses.

Macrophages are involved in almost all stages of the immune responses and play a role in the initial response to microbial infection before T- and B cell immunity are evoked. Mechanisms by which macrophages act as effector cells in a host defense system include both intracellular and extracellular cytokine secretion activities. Thus, we investigated here whether or not EYFZ could stimulate the expression and the secretion of cytokine from J774.1.

As shown in this study, EYFZ was able to induce IL-12 expression and production by J774.1. IL-12 has recently been brought into focus as an effector molecule to enhance immunity by murine macrophages\textsuperscript{48-50}. Production of IL-12 by macrophages can be induced by interaction with activated T cells, which provides costimulatory signals via molecules such as CD40 ligand. These signals appear to be essential, because their inhibition can abrogate IL-12 production. IL-12 exerts multiple effects on T and NK cells including the augmentation of IFN-\(\gamma\) production, proliferation, and cytotoxic activity, and also plays an important role to determine a Th1/Th2 balance\textsuperscript{51}. IL-12 is a heterodimeric cytokine composed of disulfide linked p40 and p35 subunits; both subunits have to be expressed within the same cell to produce biologically active p70 heterodimer\textsuperscript{52}. It has been shown that p40 mRNA expression is up-regulated in the cells producing IL-12, whereas p35 mRNA is constitutively expressed in various cells\textsuperscript{53}. As shown in Fig.4, the expressions of IL-12p35 and IL-12p40 were induced similarly as assessed at 12hrs and 24hrs after the treatment with EYFZ. Moreover, our results of ELISA show that this increased expression of IL-12p35 and IL-12p40 induced by EYFZ occurs at the protein level.

It was reported that IL-12 has anti-tumor effect\textsuperscript{54-57} and a powerful anti-tumor activity in mice against 17 different lines of transplantable murine tumors, including carcinomas, sarcomas, melanomas and lymphomas\textsuperscript{58}. IL-12 is much more effective in anti-tumor effect than other cytokines such as IL-2 and IFN-\(\alpha\), and is effective at the doses with much lower toxicity. In our study, relationship is unclear between the effects of EYFZ on anti-tumor and enhancement of NK function \textit{in vivo} and activation of macrophage-like cell line J774.1 \textit{in vitro}. However, some papers showed that components of crude drugs of EYFZ include polysaccharide\textsuperscript{59-64}, which can activate the function of macrophage. Thus, whether or not oral administration of EYFZ can induce macrophages to produce endogenous IL-12 is not clear now. So, this elucidation needs more study in the future.

In conclusion, we have found that EYFZ has the anti-tumor effects on colon-26 implanted mice via augmentation of NK cell activity and markedly activates J774.1 to produce IL-12, which is potentially an immune enhancer. Therefore, it would be important to investigate further the effective mechanism of EYFZ on the immune system.

\textbf{Acknowledgement}

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中国の伝統的漢方薬「益気養血扶正剤」の担癌マウスにおける延命とNK細胞機能に及ぼす影響並びにマウスのマクロファージJ774.1細胞のサイトカイン産生に対する影響

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（指導：赤塚 俊隆教授）

中国的伝統的漢方薬である「益気養血扶正剤」（以下EYFZと略す）の抗腫瘍活性と免疫細胞機能に及ぼす影響を知る目的で、第1にcolon-26腫瘍細胞株を移植した担癌マウスを用いて、28日間のEYFZ経口投与が担癌マウスの延命とNK細胞機能にいかなる影響を与えるかについて追究した。実験の結果、EYFZを連続的に投与された担癌マウスの寿命は非投与群と比較して有意に延命し、腫瘍サイズと体重の減少が抑制されることがわたった。一方、担癌マウスの脾臓細胞を採取して、その中のNK活性をYAC-1細胞への細胞傷害活性アセサイによって調べた結果、EYFZを投与された担癌マウスでは、非投与群と比較して有意にNK活性が高まることを明示した。第2にマクロファージ機能の影響を知る目的でマクロファージ細胞株J774.1細胞を用いてEYFZの影響をin vitroで追究した。J774.1の培養液に種々の濃度のEYFZを添加し、マクロファージ産生サイトカインへの影響をRT-PCR法及びELISA法によって調べた。実験の結果、RT-PCR法でサイトカインmRNA発現を調べると、特にIL-12p35及びIL-12p40がEYFZによってよく誘導された。またJ774.1の培養液中にIL-12産生をELISA法で検討した結果、実際にIL-12がEYFZ存在下で強く産生されることがわたった。

以上の結果から、EYFZにはcolon-26を移植された担癌マウスの延命を引き起す抗腫瘍効果があり、その作用機構の一つにNK活性の亢進とマクロファージ機能の活性化のあることが強く示唆された。

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