Effect of Maitake (*Grifola frondosa*) D-Fraction on the Control of the T Lymph Node Th-1/Th-2 Proportion

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We have already reported that the D-Fraction, a β-glucan extracted from the fruiting body of the maitake mushroom (*Grifola frondosa*), activates cellular immunity and expresses anti-tumor effects. In this study we investigated the anti-tumor functions of D-Fraction in relation to its control of the balance between T lymphocyte subsets Th-1 and Th-2. D-Fraction decreased the activation of B cells and potentiated the activation of helper T cells, resulting in enhanced cellular immunity. It also induced the production of interferon (IFN)-γ, interleukin (IL)-12 p70, and IL-18 by whole spleen cells and lymph node cells, but suppressed that of IL-4. These results suggest that D-Fraction establishes Th-1 dominance which induces cellular immunity in the population that was Th-2 dominant due to carcinoma.

Key words   polysaccharide (D-Fraction); Th-1/Th-2 cells; maitake

Tumor cells and cancer cells in the initial developmental stages are mainly scavenged by neutrophilic leukocytes, macrophages, and other components of the primitive immune system. In 1986, Mosmann and Coffman classified CD4$^+$ T cell clones into three sub-types based on cytokine production as follows: (1) helper (Th)-1 cells, that produce interleukin (IL)-2, interferon γ (IFN)-γ, and tumor necrosis factor (TNF)-β, and introduce cellular immunity to the organism; (2) Th-2 cells, that produce IL-4, IL-5, IL-6, IL-10 and Il-13, and activate humoral immunity; and (3) precursor or Th-0 cells, that produce IL-4 and IFN-γ concomitantly.[1,2] Cytokine derived from Th-1 cells activates cytotoxic T cells mainly, and enhances delayed type hypersensitivity, while cytokine derived from Th-2 cells stimulates antibody production. The immune response is chosen depending on which sub-type of T cell is activated, which means that the proportion of the activated sub-types influences phylaxis immunity and anti-tumor immunity. This control system is also affected by the production of IL-1β, IL-12, and IL-18 by antigen presenting cells (APC).[3,4] Recent study shows that many immune disorders are attributable to the collapse of the system controlling the proportion of Th-1 to Th-2 cells.[5] Restoration of the proper balance between Th-1 and Th-2 cells is perceived as essential in the treatment of tumors, which are generated when cellular immunity is affected by immuno-suppressing factors.

We have already reported that the D-Fraction, a β-glucan extracted from the fruiting bodies of the maitake mushroom (*Grifola frondosa*), activates cellular immunity and expresses anti-tumor effects.[6—8] However, the mechanism by which cytokine, the humoral factor of the immunity, provokes tumor-rejection during the anti-tumor action of D-Fraction, remained to be investigated. In this study, we investigated the change in the proportion of Th-1/Th-2 cells when maitake mushroom D-Fraction expresses its anti-tumor effects, and also how the cytokines are involved in the mechanism.

MATERIALS AND METHODS

Materials   A dried powder made from the fruiting bodies of the maitake mushroom (*Grifola frondosa*) was obtained from Yukiguni Maitake Co., Ltd. (Niigata, Japan). Mouse IL-1β, IL-12 p70, IL-18, and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Genzyme Co. (Minneapolis, MI, U.S.A.). Cytofix/Cytoperm Plus (with Golgi Stop^{TM}) kit was purchased from Parmingen Co. (San Diego, CA, U.S.A.).

Antibodies   The following monoclonal antibodies were purchased from Pharmingen Co.: CD16/CD32 (0.5 mg/ml), FITC-conjugated CD8 (0.5 mg/ml), FITC-conjugated CD69 (0.5 mg/ml), FITC-conjugated IFN-γ (0.5 mg/ml), R-PE-conjugated IL-4 (0.2 mg/ml), R-PE-conjugated CD19 (0.2 mg/ml), R-PE-conjugated CD69 (0.2 mg/ml), Cy-Chrom^{TM}-conjugated CD3ε (0.2 mg/ml), and Cy-Chrom^{TM}-conjugated CD4 (0.2 mg/ml).

Animals   Male C3H/HeN mice (4 weeks old) were provided by Japan Clea Co. (Osaka, Japan) and were raised for one week before being used for experiments.

The D-Fraction was prepared from the dried powder according to a method described previously.[9]

Dosage of D-Fraction   MM-46 carcinoma cells (2×10^6) were implanted in 8 male mice of the C3H/HeN strain (5 weeks old) in the right axillary region. After 24 h, the D-Fraction ($5\,\mathrm{mg \cdot kg^{-1} \cdot d^{-1}}$) was administered to the MM-46 carcinoma-bearing mice intraperitoneally (i.p.) for 19 consecutive days. As a control, phosphate buffered saline (PBS) was also injected for 19 consecutive days. This trial was done 3 times, and the same results were obtained each time.

Preparation of Whole Spleen Cells   On day 20 in mice administered D-Fraction or PBS, the spleen was extirpated from any 2 mice in 5 mice of the treated group, passed through nylon mesh ($\phi70\,\mu\mathrm{m}$), and washed with RPMI-1640 medium by centrifugation ($300 \times \boldsymbol{g}$, 5 min, 4 °C). The precipitated cells were collected and hemolyzed to remove erythrocytes. After the centrifugation, the precipitated cells were washed with RPMI-1640 medium and suspended in RPMI-1640 medium containing 10% fetal bovine serum (FBS).

Preparation of Lymph Node Cells   On day 20 after the administration of D-Fraction or PBS, the inguinal lymph node was extirpated from any 2 mice in 5 mice of the treated group, passed through nylon mesh, and washed with RPMI-1640 medium by centrifugation ($300 \times \boldsymbol{g}$, 5 min, 4 °C). The

precipitated cells were collected and suspended in RPMI-1640 medium containing 10% FBS.

Preparation of Peritoneal Macrophages On day 20 after the administration of D-Fraction or PBS, 3 mice were killed by vertebral dislocation, and the cells were obtained by washing the peritoneal cavity with RPMI-1640 medium. After centrifugation ($300 \times g$, 5 min, 4 °C), the precipitated cells were suspended in RPMI-1640 medium containing 10% FBS. After cultivation, adherent cells were collected.

Determination of Cytokine Release by ELISA One times 10^5 cells/well of whole spleen cells or lymph node cells obtained from 3 mice in 5 mice of the treated group were cultured in a 96-well plate with Con A (final concentration, 10 μg/ml) at 37 °C for 24 h in 5% CO_2. After the stimulation, the culture supernatant (100 μl) was collected by centrifugation ($300 \times g$, 5 min), and levels of IL-12 p70, IL-18, and TNF-α were determined by ELISA. IL-1β in the culture supernatant of peritoneal macrophages (1×10^5 cells/well) without Con A was also determined by ELISA.

Flow Cytometry[10] For cell-surface antigen detection, 100 μl of the whole spleen cells or lymph node cells (1×10^7 cells/ml) was mixed with 1 μl of anti CD16/CD32 monoclonal antibody in a tube and reacted at 4 °C for 5 min. The cells were incubated with FITC-conjugated monoclonal antibodies (anti-CD8, anti-CD69), R-PE-conjugated monoclonal antibodies (anti-CD19, anti-CD69), or Cy-Chrome™-conjugated monoclonal antibodies (anti-CD3ε, anti-CD4) (mAbs$\geqq$ 1 μg) at 4 °C for 35 min, washed with Washing Solution (0.09% NaN_2 and 1% FBS in PBS), then suspended in 0.5 ml of Washing Solution and enumerated with a FACScan™ flow cytometer (Beckton Dickinson, Grenoble, France).

For intracellular cytokine detection, 1 ml of the whole spleen cells or lymph node cells (2×10^6 cells/ml) was applied to a 24-well plate, ionomycin (750 ng/ml) and phorbol-12-myristate-13-acetate (50 ng/ml) were added to each well, and the plate was incubated with 1.4 μl of Goldi Stop at 37 °C in 5% CO_2 for 4 h. After the stimulation, 0.5 μl of anti CD16/CD32 monoclonal antibody was added and reacted at 4 °C for 5 min. Then, each Cy-Chrome™-conjugated CD4 monoclonal antibody (mAbs$\geqq$1 μg) was reacted at 4 °C for 20 min. After the reaction, the cells were washed with Staining Buffer (0.09% NaN_2 and 2% FBS in PBS), incubated with 100 μl of Cytofix/Cytoperm at 4 °C for 20 min, and washed again with Perm/Wash. The stained cells were reacted with each of the FITC-conjugated IFN-γ monoclonal antibodies (0.2 μg) and R-PE-conjugated IL-4 monoclonal antibodies (0.1 μg) for CD4$^+$ T cells at 4 °C for 30 min, then washed with Perm/Wash, suspended in 0.5 ml of Staining Buffer, and counted by FACScan™. Cells were obtained from any 3 mice in 5 mice of the treated group.

Statistical Analysis Values are expressed as the mean$\pm$ S.E.M, and differences between the control mice and the D-Fraction-administered mice were examined using Student's t-test.

RESULTS

Activation of T Cells and B Cells As shown in Fig. 1, relative to the control mice, the D-Fraction-administered mice exhibited a tumor inhibition rate (T.I.R.) of 82%. The significant inhibition by D-Fraction was presumed to activate
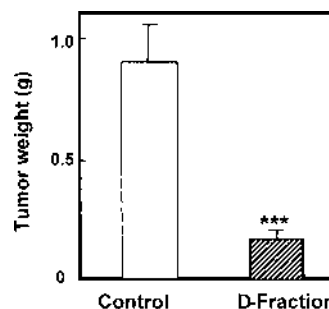

Fig. 1. Effect of D-Fraction on MM-46 Carcinoma Cells

MM-46 carcinoma cells (2×10^6) were implanted in 8 male mice of the C3H/HeN strain (5 weeks old) in the right axillary region. After 24 h, the D-Fraction (5 mg·kg^{-1}· d^{-1}) was administered to the MM-46 carcinoma-bearing mice i.p. for 19 consecutive days. On day 20, tumor weight was measured. Data are the means$\pm$S.E.M. of 8 different experiments. ***$p < 0.001$ compared with the control.
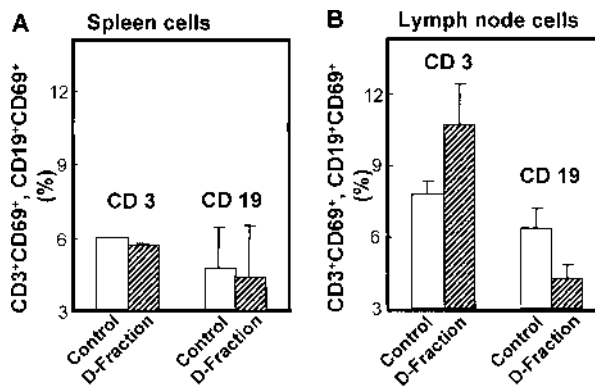

Fig. 2. Effects of D-Fraction on CD3$^+$ and CD19$^+$ Cell Activation in Whole Spleen Cells (A) or Lymph Node Cells (B) Obtained from Any 2 Mice of 5 Mice in the Group

Data are the means$\pm$S.E.M. of 3 different experiments.

lymphocytes which enhance anti-tumor immunity. We investigated, therefore, the activation of T cells and B cells in the whole spleen and in the lymph node of the inguinal region of D-Fraction-administered mice bearing MM-46 carcinoma for 20 d. The target for detection was CD3ε^+ in the T cells, and CD19$^+$ in the B cells, surface antigens of the respective cell populations. CD69 is known to be the early activation marker on CD4$^+$ T cells, CD8$^+$ T cells and B cells, in addition to macrophages and NKT cells.[11] To study the activation of these cells, we also determined the expression ratio of CD69 by flow cytometric analysis. As shown in Fig. 2A, there was no effect on B cell or T cell activation by D-Fraction in the whole spleen cells. In the inguinal lymph node, however, CD69 expression in T cells increased compared with the control mice (Fig. 2B). On the other hand, CD69 expression in B cells decreased. Rates of activated T cell/B cell increased 2-fold in the D-administered mice. These results suggest that D-Fraction inhibits the introduction into the tumor area of B cells which produce antibodies, but activates T cells and stimulates cellular immunity.

Activation of T Cell Subsets To clarify which of the T cell subsets contributes to the cellular immunity, we prepared CD4$^+$ cells and CD8$^+$ cells as targets because they are the surface antigens for helper T cells (Th) and cytotoxic T cells (CTL), respectively. As shown in Fig. 3, in the inguinal lymph node, CD69 expression in CD4$^+$ cells increased com-

pared with the control, and CTL expression increased slightly. The proportion of activated CD4$^+$ to CD8$^+$ cells increased 2.2-fold by administration of D-Fraction. These results indicate that the administration of D-Fraction to tumor bearing mice activates CD4$^+$ among the T cell subsets.

IFN-γ or IL-4 Expression in CD4$^+$ T Cells Th cells are classified into two sub-types, Th-1 cells which produce IL-2 and IFN-γ and stimulate cellular immunity, and Th-2 cells which release IL-4, IL-5, IL-6, and IL-10 and activate humoral immunity.[1,2] As the cytokines produced by these two sub-types have different immunological activities, the balance between the two is considered to be important to antitumor immunity. Therefore, we investigated the cytokines expressed in the CD4$^+$ T cells. We analyzed the IFN-γ and IL-4 expression ratio in the CD4$^+$ T cells using flow cytometry by setting up a lymphocyte gate for whole spleen cells and inguinal lymph node cells, and also a gate for CD4$^+$ cells (Fig. 4). The productivity ratio for IFN-γ increased 1.4-fold compared with the control value, while that for IL-4 was unchanged. The expression ratio of IL-4 in the inguinal lymph node cells was 0.7 for D-administered mice. The expression ratio of IFN-γ was increased at 1.6-fold.
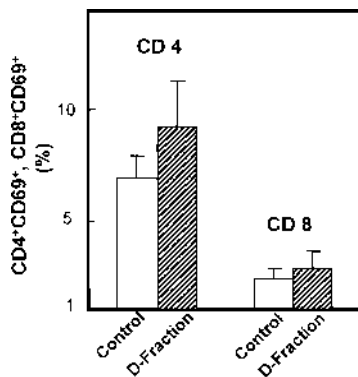
This result was obtained by using of any 3 mice in 5 mice of the treated group.

IL-12 and IL-18 Production by Whole Spleen Cells and Lymph Node Cells On tumor initiation, various cytokines are released from immuno-competent cells, other than from CD4$^+$ T cells. To confirm this, we investigated IL-12 p70, IL-18, IL-1β and TNF-α, which would affect the Th-1/Th-2 population. Whole spleen cells and inguinal lymph node cells were stimulated by cultivation for 24 h, and the cytokines in the culture supernatant were measured by ELISA. In both populations of cells, the production of IL-12 p70 increased significantly compared with the control (Fig. 5A). IL-12, which is released from Th-1 cells, is an important cytokine that promotes cellular immune reactions, and stimulates IFN-γ productivity in T cells.[12] IL-12 p70 production increased because D-Fraction activated immunity in the dominant Th-1 population. As shown in Fig. 5B, D-Fraction increased IL-18 production in the whole spleen cells by 1.7-fold, and in the inguinal lymph node cells by 4.7-fold compared with the control. IL-18 strongly induces the production of IFN-γ by T cells and B cells.[13] Insert T cells do not respond to IL-12 or IL-18, but when primed by a carcinoma antigen, the cells become responsive to IL-12 or IL-18 and produce IFN-γ. The amount of IL-1β in the supernatant obtained from peritoneal macrophages cultured for 24 h was examined. As shown in Fig. 6, a marked decrease in IL-1β was observed in the D-Fraction-administered mice, to 0.4 of the control. IL-1β is an inflammatory cytokine produced mostly by antigen presenting cells such as macrophages, and acts as a secondary stimulant for Th-2 activation. Mouse Th-1 cells do not express IL-1β receptors, therefore, the cells are not affected by this cytokine.[14] The decrease in IL-1β production indicates that D-Fraction inhibited the stimulation of Th-2 cells. Figure 7 shows TNF-α production by whole spleen cells. D-Fraction-administered mice exhibited a 2.5-fold increase in production compared with the control. TNF-α is a cytokine mainly produced by macrophages, and has various functions in relation to inflammatory and cytotoxic reactions, as well as cytotoxicity, and directly causes hemor-
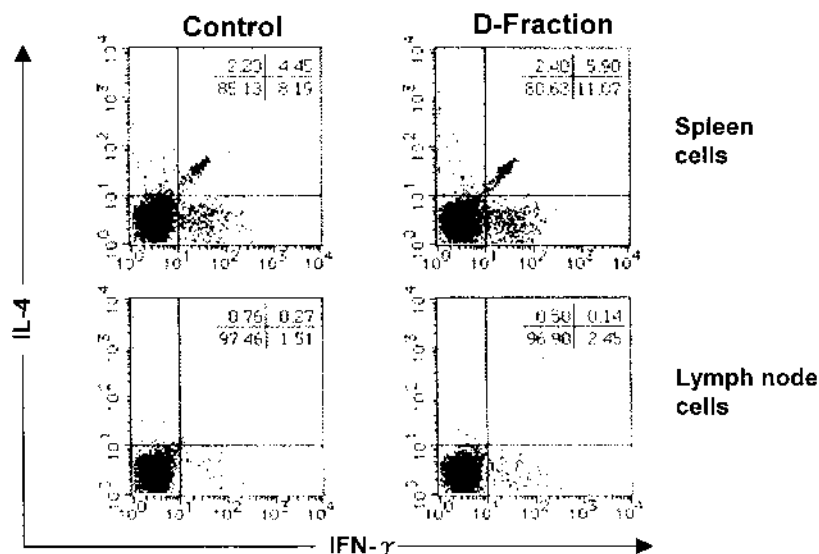

Fig. 3. Effects of D-Fraction on CD4$^+$ and CD8$^+$ Cell Activation in Lymph Node Cells Obtained from Any 2 Mice of 5 Mice in the Group

Data are the means±S.E.M. of 3 different experiments.

Fig. 4. Effects of D-Fraction on IFN-γ Expression and IL-4 Expression in Whole Spleen Cells or Lymph Node Cells Obtained from Any 3 Mice of 5 Mice in the Group
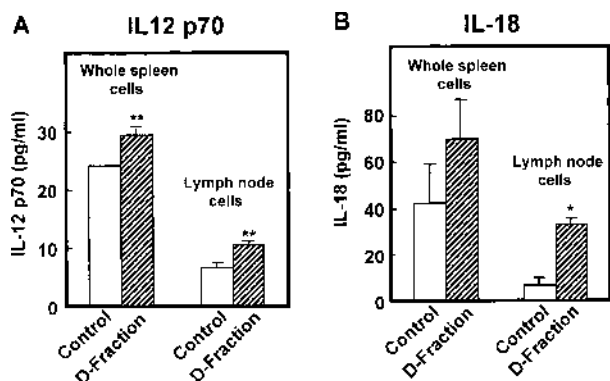
Fig. 5. Effects of D-Fraction on IL-12 p70 Production (A) or IL-18 (B) Production by Whole Spleen Cells or Lymph Node Cells Obtained from Any 3 Mice of 5 Mice in the Group

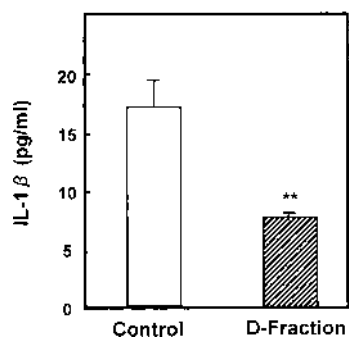Data are the means±S.E.M. of 4 different experiments. $*p<0.05$ and $**p<0.01$ compared with the control.

Fig. 6. Effect of D-Fraction on IL-1β Production by Peritoneal Macrophages Obtained from Any 3 Mice of 5 Mice in the Group

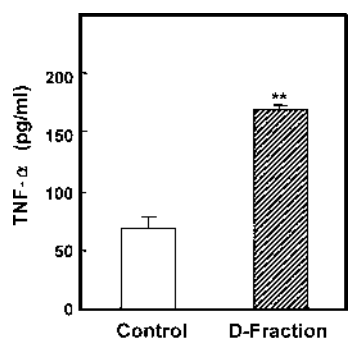Data are the means±S.E.M. of 4 different experiments. $**p<0.01$ compared with the control.

Fig. 7. Effect of D-Fraction on TNF-α Production by Whole Spleen Cells Obtained from 3 Mice of 5 Mice in the Group

Data are the means±S.E.M. of 4 experiments. $**p<0.01$ compared with the control.

rhagic tumor necrosis. Consequently, the significant increase in TNF-α production indicates that D-Fraction activated macrophages. It also indicates that the anti-tumor effects of D-Fraction are partly attributable to enhanced production of TNF-α.

DISCUSSION

D-Fraction demonstrated a significant anti-tumor effect when administered i.p. This effect is presumed to be caused mainly by the lymphocytes that introduce tumor-specific immunity, and not by non-specific immune reactions. We examined the change in the relative proportion of activated T cells to B cells in the spleen and in the inguinal lymph node. T cell activation by D-Fraction was observed in the inguinal region, which is closer to the carcinoma than the spleen. This suggested that the T cells activated by D-Fraction were delivered to the tumor area from the spleen. Also, it indicated that D-Fraction in carcinoma-bearing individuals triggered cellular immunity by inhibiting antibody production by B cells and activating T cells in the tumor area. Our aim was to find out which subset of T cells was activated by D-Fraction. D-Fraction induced significant differentiation, multiplication, and enhancement of the activation of CD4$^+$ cells. This result suggests an introduction of cellular immunity by the CD4$^+$ population. D-Fraction also weakly enhanced CTL activation. This seems to be related to the activation of CTL by the activated CD4$^+$ cells. The CD4$^+$ T cells are classified into two sub-types based on cytokine production, that is, Th-1 cells which produce IL-2, IFN-γ and TNF-β, and evoke cellular immunity, and Th-2 cells which produce IL-4, IL-5, IL-6, L-10, and IL-13, and stimulate humoral immunity. The balance between these two sub-types is presumed essential to phylaxis immunity, anti-tumor immunity, etc. To verify this, flow cytometry was used to obtain an expression ratio of two intracellular cytokines, IFN-γ and IL-4 in CD4$^+$ T cells. The results indicate that IFN-γ expression increased and IL-4 expression decreased when D-Fraction was administered to tumor-bearing mice. This suggests that D-Fraction established Th-1 dominance in a T cell population which was once Th-2 dominant due to carcinoma. In addition, an investigation was also made on IL-12 p70 and IL-18, the cytokines controlling the Th-1/Th-2 balance. IL-12 is a heterodimer of P35 and P40,[13,14] and activates macrophages and NK cells by promoting immune responses directed by Th-1. IL-18, formerly known as IGIF (IFN-γ inducing factor),[15,16] cooperates with IL-12 to strongly promote the production of IFN-γ by T cells, and B cells, and also enhances the functions of Th-1 cells as well as promoting the expression of Fas ligand.[13,14] However, recent findings show that IFN-γ is produced only when IL-12 coexists with IL-18; otherwise, T cells, basofilic leukocytes, mastcytes, etc. are acted upon, and Th-2 cytokines such as IL-4/IL-13 are produced in vitro.[17] In addition, IL-18 stimulates CD4$^+$ T cells in vitro and induces the production of IgE dependent on IL-4.[18] In this investigation, we found that production of IL-12 p70 and IL-18 was enhanced in whole spleen cells and in the inguinal lymph node, which suggests that D-Fraction evokes Th-1-dominant immune reactions even in carcinoma-bearing mice. This result also explains why the production of IFN-γ was enhanced by D-Fraction. It also suggests that the Th-1/Th-2 population became Th-1 dominant owing to the inhibition of IL-1β production in peritoneal macrophages by the effect of D-Fraction. As stated above, D-Fraction can be used to control the balance between Th-1 and Th-2 cells. However, the mechanism by which naive cells (Th-0) achieve functional conversion to become Th-1 or Th-2 cells is yet to be clarified. Th-1 cells have the ability to change temporarily into Th-2 cells, but mature Th-2 cells cannot change back. This is because Th-1 cells are affected by IL-4 signals even after attaining maturity, but Th-2 cells, when mature, do not recognize IL-

12 signals.[19] Therefore, the function of D-Fraction in establishing Th-1 dominance is not the changing of mature Th-2 into Th-1 cells, but more likely either the enhancement of conversion of naive cells, *i.e.* Th-0 into Th-1 cells, or the inhibition of conversion of Th-1 into Th-2 cells.

In summary, the maitake D-Fraction induces the onset of immunity dominated by Th-1 cells converted from Th-0 cells, and consequently stimulates cellular immunity, and expresses anti-tumor effects.

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