T CELL ACTIVATION BY CONAGENIN IN MICE

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Conagenin (CNG), a low molecular immunomodulator, enhanced incorporation of [^{3}H]thymidine into T cells activated by concanavalin A but did not to non-activated T cells. The culture supernatants of activated T cells treated with CNG enhanced incorporation of [^{3}H]thymidine into cytokine dependent cell lines, CTLL-2 and IC-2 cells. This indicates that CNG exclusively acts on activated T cells and stimulates them to promote DNA synthesis and to produce lymphokines, which may include T cell growth factors and hematopoietic growth factors. These activities were also observed with T cells taken from mice given CNG.

Immunomodulation is now an important therapeutic strategy for treatment of immunodeficient diseases such as cancer, transplantation and other immunological disorders. Most immunomodulators like MDP[1], ubenimex[2] and forphenicinol[3,4] act primarily on macrophages and consequently stimulate T cells. However, it is known that macrophage activation enhances inflammatory responses which frequently down regulates diseases. Thus, we have attempted to search for an immunomodulator exhibiting specific action on T cells in microbial products and found conagenin (CNG). CNG was isolated from cultured broth of *Streptomyces roseosporus* as an immunomodulator which enhances [^{3}H]thymidine incorporation of T cells activated by concanavalin A (Con A)[5]. In this study, we investigated the effects of CNG on [^{3}H]thymidine incorporation into T cells and their lymphokine production.

Materials and Methods

Mice

CDF_1 mice (6 weeks old, female) were purchased from Charles River Japan Inc. (Kanagawa, Japan) and were maintained under specific pathogen-free conditions at 23 ± 2°C and 55 ± 5% humidity. They were employed for experiments at 9 to 11 weeks of age.

CNG

CNG was prepared by KANEKA Co., Ltd. (Osaka, Japan), according to the methods reported previously[5], and dissolved in culture medium and saline.

Cell Preparation

Murine spleen cells passed through a nylon wool column (Wako Chemical Co. Ltd., Tokyo, Japan) were used as T cells. In the following experiments *in vitro*, non-activated T cells (NATC) and Con A-activated T cells (CATC) were employed. CATC were prepared as follows; T cells (5×10^6 cells/ml in RPMI 1640 (Nissui Seiyaku Co. Ltd., Tokyo, Japan) containing 10% heat inactivated fetal calf serum) were incubated with Con A (2 μg/ml: Pharmacia Fine Chemical Inc., Uppsala, Sweden) at 37°C in 5% CO_2 for 4 hours and were washed thoroughly with α-methyl mannoside (20 mg/ml: Sigma, St. Louis, U.S.A.).

To obtain macrophages, peritoneal exudate cells (PEC) were collected from mice and 1 ml of PEC at 1×10^6 cells/ml in RPMI 1640 were plated, then incubated at 37°C in 5% CO_2 for 1 hour. After washing

with RPMI 1640, adherent cells ($4 \sim 5 \times 10^5$ cells/plate) on the bottom of the plate were used as macrophages.

Assay for Incorporation of [^{3}H]Thymidine into T Cells

NATC and CATC were treated with CNG at 37°C in 5% CO_2 for 4 hours. After washing with RPMI 1640 thoroughly, these cells were suspended at 2.5×10^6 cells/ml in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum, 50 μM 2-mercaptoethanol, non-essential amino acids, 1 mM sodium pyruvate, 50 units/ml penicillin and 50 μg/ml streptomycin, and were put in wells of a microplate (0.2 ml/well) and cultured for 3 days. Eighteen hours before termination of culture, [^{3}H]thymidine ([^{3}H]TdR: [6-^{3}H]thymidine, sp. act. 555GBq/mmol, NET-355, New England Neuclear, Boston, U.S.A.) was added (7.4 KBq/well) and its incorporation into cultured cells was counted by a liquid scintillation counter. Triplicate cultures were made for each determination.

Assays for Cytokine Activities

CATC or NATC (5×10^6 cells/ml) were treated with CNG for 4 hours and were cultured at 37°C in 5% CO_2 for 3 days. After incubation, the culture supernatants were collected and stored at −80°C until assay.

In order to determine effects of CNG on monokine production by macrophages, adherent PEC on the plates were treated with CNG at 37°C in 5% CO_2 for 4 hours. After washing, PEC were cultured for 1 day, the culture supernatants were collected and stored at −80°C until assay.

The cytokine activities in the culture supernatants were measured by incorporation of [^{3}H]TdR into cultured cytokine dependent cell lines. D10.G4.1[6], CTLL-2[7] and IC-2[8] cells were used as target cells. The target cells (1×10^4 cells/well) were cultured with 100 μl of the T cell culture supernatants at 37°C in 5% CO_2 for 3 days for D10.G4.1. cell cultures or 2 days for CTLL-2 and IC-2 cell cultures. The cultured cells were pulsed with [^{3}H]TdR (7.4 KBq/well), 18 hours before assay, and the incorporation of radioactivity into cells was counted by a liquid scintillation counter. Triplicate cultures were made for each determination.

Effect of CNG in Mice

CNG at 5 mg/kg was given ip to mice, splenic T cells and adherent PEC were prepared as described above from mice on days 1 to 7 after administration. T cells and adherent PEC were cultured at 37°C in 5% CO_2 for 3 days or 1 day, respectively. [^{3}H]TdR incorporation into and lymphokine production of T cells and monokine production of PEC were determined. Five mice were used for an assay.

Statistical Analysis

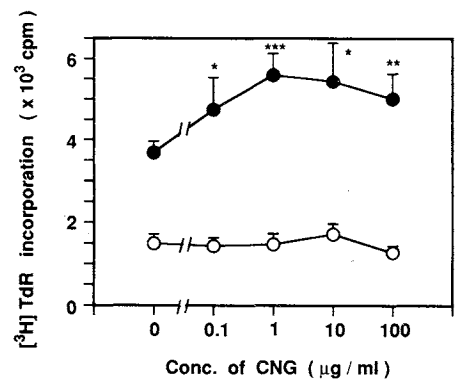
Statistical significance was analyzed by STUDENT's *t*-test.

Results

The effect of CNG on [^{3}H]TdR incorporation into CATC and NATC was investigated. Both cells were treated with CNG for 4 hours and CNG was washed out. Then, cells were cultured for 3 days and the incorporation of [^{3}H]TdR into cultured cells was determined. As shown in Fig. 1, [^{3}H]TdR incorporation into CATC treated with CNG at 0.1

Fig. 1. Effect of conagenin (CNG) on [^{3}H]thymidine incorporation into murine splenic T cells.

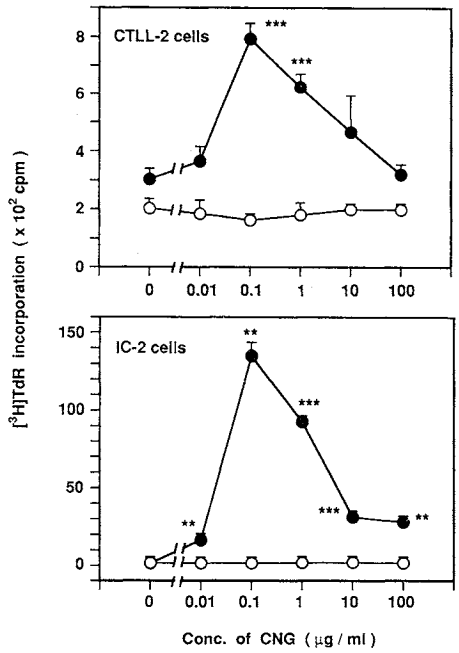
○ Non-activated T cells (NATC), ● concanavalin A-activated T cells (CATC).



CATC: splenic T cells treated with Con A for 4 hours. NATC: non treated T cells. CATC and NATC were treated with CNG for 4 hours. After washing, these cells were cultured for 3 days. [^{3}H]TdR incorporation into cells was measured. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in comparison with cultures without CNG.

Fig. 2. Lymphokine activities in culture supernatants of splenic T cells treated with conagenin (CNG).

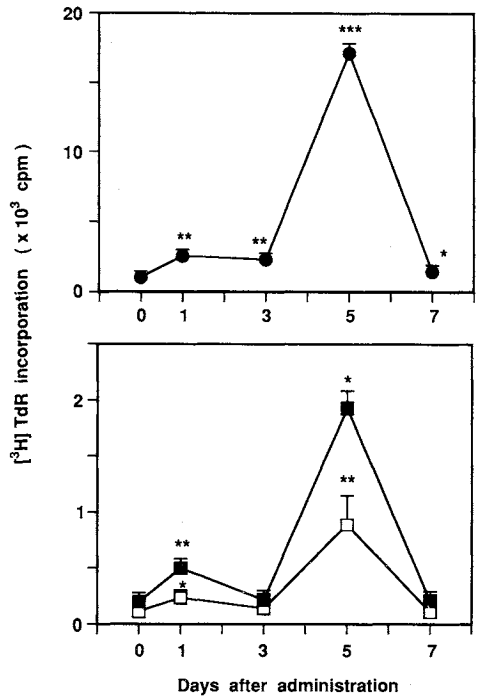
○ Non-activated T cells (NATC), ● concanavalin A-activated T cells (CATC).



CATC and NATC were treated with CNG for 4 hours. After washing, these cells were cultured for 3 days. CTLL-2 and IC-2 cells were cultured with the T cell culture supernatants for 2 days. [^{3}H]TdR incorporation into CTLL-2 and IC-2 cells was measured. ** $P<0.01$ and *** $P<0.001$ in comparison with cultures without CNG.

Fig. 3. [^{3}H]Thymidine incorporation into splenic T cells taken from mice given conagenin (CNG) and their lymphokine production.

● T cells, ■ IC-2 cells, □ CTLL-2 cells.



Splenic T cells taken from mice given CNG ip at 5 mg/kg were cultured for 3 days. [^{3}H]TdR incorporation into these T cells was measured. CTLL-2 and IC-2 cells were cultured with T cell culture supernatants for 2 days. [^{3}H]TdR incorporation into CTLL-2 and IC-2 cells was measured. * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ in comparison with day 0.

to 100 μg/ml was significantly enhanced. The enhancement of incorporation was also observed in cultures of CATC treated with CNG for 8 hours, but did not in CNG treatment for 2 hours and 2 to 3 days. In any case, CNG did not enhance [^{3}H]TdR incorporation into NATC. On the other hand, the addition of CNG for 3 consecutive days to lymphocyte cultures containing Con A or LPS did not enhance mitogenic activity of these lectins.

Next, the effect of CNG on lymphokine production was investigated. CATC or NATC were treated with CNG for 4 hours and cultured for 3 days. As shown in Fig. 2, the supernatants taken from CATC treated with CNG enhanced the incorporation of [^{3}H]TdR into CTLL-2 cells at 0.1 to 1 μg/ml and IC-2 cells at 0.01 to 100 μg/ml but the supernatants from NATC did not. These results demonstrate that CNG acts on CATC exclusively.

The effect of CNG on T cell functions was investigated in mice. After administration of CNG ip to mice, splenic T cells were prepared and the incorporation of [^{3}H]TdR into cultured cells and lymphokine activities of the culture supernatants were determined. As shown in Fig. 3, the [^{3}H]TdR incorporation into T cells increased on days 1 to 7 after administration and the culture supernatants prepared from T

cell cultures enhanced the incorporation or [^{3}H]TdR into CTLL-2 and IC-2 cells on days 1 and 5. A marked effect was observed on day 5 in both assays. These results indicate that CNG acts on T cells to stimulate incorporation of [^{3}H]TdR and production of lymphokines *in vitro* and *in vivo*.

On the other hand, the culture supernatants of PEC treated with CNG at 0.1 to 100 μg/ml and of PEC taken from mice given CNG did not enhance the incorporation of [^{3}H]TdR into D10.G4.1. cells. It should be noted that CNG was not able to induce and to enhance monokine production by PEC *in vitro* and *in vivo*.

Discussion

Most immunomodulators such as MDP[1], ubenimex[2] and forphenicinol[3,4] are known to be activators for macrophages. It is obvious that afferent and/or efferent phases in the immune system are conducted by T lymphocytes. Thus, we have attempted to search for an immunomodulator which modulates T cell functions primarily. Accordingly, CATC which are activated with Con A have been employed for screening and CNG was found in microbial products[5]. As shown above, CNG stimulated [^{3}H]TdR incorporation into CATC but did not stimulate NATC. The culture supernatants of CATC treated with CNG also stimulated [^{3}H]TdR incorporation into lymphokine dependent cell lines, CTLL-2 and IC-2 cells. Although the identification and quantitation of lymphokines in the supernatants were not determined, it is considered that CNG enhances production of lymphokines such as IL-2, IL-3, IL-4 and GM-CSF by activated T cells, since CTLL-2 cells are used to assess murine T cell growth factor activity including IL-2 and IL-4[7,9] and IC-2 cells are used for hematopoietic cell growth factor assays including IL-3 and GM-CSF[10]. These effects of CNG were also demonstrated in mice.

It will be reported that we investigated the binding activity of CNG to T cells treated with or without Con A and EL-4 thymoma cells and found that CNG only bound to T cells activated by Con A or EL-4 cells but did not to non-activated T cells[11]. The binding study suggests that the activated T cells may have a specific binding site for CNG.

It is reported that ubenimex also stimulates the proliferation of T cells[2]. However, ubenimex activates macrophages to induce production of IL-1[2,12]. In this connection, CNG did not activate peritoneal macrophages to produce monokine. We found that the administration of CNG reduces monokine production by peritoneal macrophages in tumor bearing mice[13].

From results mentioned above, a low molecular immunomodulator CNG may be useful for cancer treatment and other immunological disorders.

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