EFFECT OF GINSENG ON THE IMMUNE RESPONSES TO INFLUENZA VIRUS INFECTION IN MICE

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Abstract

Total saponins extracted from *Panax ginseng* have no effect on the cytotoxic T-cell activity, and natural killer cell activity in mice infected with A/WSN influenza virus. The saponins, however suppressed delayed-type hypersensitivity responses to the virus and to sheep erythrocytes when administered to the animal before sensitization.

Thus a prophylactic anti-inflammatory action of the total saponins of ginseng is observed, which may be related to their steroid-like structure.

Introduction

Ginseng has been highly valued as a "cure-all" drug by the orientals for thousands of years. Many constructive effects of the herb have been studied experimentally such as adaptation to high and low temperatures, anti-fatigue and anti-stress effects, skin regeneration and anti-wrinking, anti-toxic and anti-ageing, stimulation of carbohydrate and lipid metabolism, hyper- and hypotensive effects, hyper- and hypothermia effects(Li & Li, 1973; Popov & Goldwag, 1973). The present investigations aim at testing whether the total saponins of *Panax ginseng* roots can in some way affect or modulate the immune responses of mice to influenza virus infection, a well known immunopathological and immunopharmalogical model. Aspects of the immune response studied include cytotoxic T-cell (Tc) activity, natural killer cell (NK) activity, and delayed-type hypersensitivity (DTH).

Experimental Procedures

Ginseng saponins

Dried roots of *Panax ginseng* was cut into small pieces and refluxed with 90% MeOH. Distilled water was added to the concentrated MeOH extract, which was then extracted with petroleum ether in a separation funnel. The aqueous fraction after separation was extracted with water-saturated n-BuOH. The BuOH fraction contained the total saponins of ginseng and was used in all subsequent experiments. The saponins were dissolved in PBS and injected intravenously into mice.

Virus

Influenza virus strain A/WSN(HON1) was grown in the allantoic cavity of 10-d-old embryonated eggs for 40–48 h. The infectious allantoic fluid was stored at −70° C until use. Virus titres are expressed as hemagglutinin units (HAU). The virus was purified as described by Braciale & Yap (1978) and was UV inactivated according to Leung, Ada & McKenzie (1980).

Tumor cells

P815, RBL5 and YAC murine tumor cells were maintained in Dulbecco's modified medium (H-16, Grand Island Biological Co.) supplemented with 10% inactivated foetal calf serum and containing 60 μg/ml of penicillin G, 100 μg/ml of streptomycin sulphate and 100 μg/ml of neomycin sulphate (these antibiotics are together referred as PSN). The cultures were kept at 37° C under a gas phase of 10% CO_2 in air. The cells were cultured at an initial concentration of 10^5 cells/ml and subcultured at 2 d intervals when the cell concentration was about 10^6 cells/ml.

Cytotoxic T-cell assay

Each mouse (CBA, 7–12 wk old) was given intravenously 10^3 HAU of infectious A/WSN influenza virus. The virus was suspended in 0.3 ml of phosphate-buffered saline (PBS). Six days after the infection, cytotoxicity in the splenic lymphocytes was measured as follows. 5×10^6 P815 cells in 0.3 ml culture medium (F-15, Grand Island Biological Co., supplemented with 10% inactivated foetal calf serum and contained PSN) were labelled with 500 μCi ^{51}Cr (sodium chromate, 200 mCi/mg; CEA commissariat, A L'energie Atomique, Siren) at 37° C for 1 h. After labelling, the cells were washed twice with the medium and then divided into two equal lots. One lot was infected with stock A/WSN virus (2.5×10^6 labelled cells infected with 250 μl virus) and the other lot uninfected and used as control. These target P815 cells were then incubated with splenic lymphocytes (effector cells) isolated from the experimental mice. Normally the effector cells were prepared in culture medium at concentrations of 5×10^6, 1×10^7 and 2×10^7 cells/ml. The target cells were prepared at 1×10^5 cells/ml so that when 0.1 ml of each effector and target cells were incubated together in multi-dish disposo trays (Linbro Scientific Co.), the effector to target cells ratios were 25:1, 50: 1 and 100:1. The incubation was at 37° C under a gas phase of 10% CO_2 in air for 6h, after which 0.1 ml of the the supernatant of the incubation mixture was pipetted out from each well of the tray and counted for radioactivity. For spontaneous lysis, 0.1 ml medium was added to 0.1 ml of target cells. For total releasable ^{51}Cr, 2.0 ml distilled water was added to 0.1 ml of labelled target cells and left on bench for 6 h. This was then spun down and the supernatant was sucked up and counted for radioactivity. The percent specific lysis was calculated as: % specific lysis =

$$\frac{\text{Test culture counts} - \text{Spontaneous release counts}}{\text{Water lysis counts} - \text{Spontaneous release counts}}$$

Natural killer cell assay

The CBA mice were treated with ginseng and/or A/WSN virus as mentioned in the text. Two days after the treatments, effector cells and target cells were prepared as in cytotoxic T-cell assays except that the target cells (RBL5 and YAC) were all uninfected with the virus. The percent specific lysis was calculated in the same way as mentioned above.

Measurement if delayed-type hypersensivity in mice

DTH in mice was determined by measuring footpad swelling (Leung *et al.*, 1980). The animal was first sensitized by injecting subcutaneously 0.3 ml (= 10^3 HAU) of infectious A/WSN influenza virus (0.15 ml on each side of the animal at a position just above the leg). Six days later the mouse was challenged with 30 μl (= 6×10^3 HAU) UV irradiated virus, injected subcutaneously into the right-hind footpad. PBS was injected into the left-hind footpad as control. The footpad thickness was measured 24, 48 and 72 h later with a pair of calipeers (H.C. Kröplin, Schlüchtern, Hessen, Federal Republic of Germany, calibrated to 0.05 mm). The increase in footpad thickness was calculated as the difference of readings (right-hand side vs. left-hand side), divided by the mean thickness of the footpad measured before challenge.

For DTH to sheep erythrocytes (SRBC) (Mitsuoka, Teramatsu, Baba, Morikawa & Yasuhira, 1978), the mouse was first sensitized intravenously

with 10^6 SRBC (in 0.3 ml of PBS). Four days later, the animal was given a subcutaneous challenge injection of 10^8 SRBC (in 30 μl PBS) into the right-hind footpad. PBS was injected into the left-hind footpad and the increase in footpad thickness was measured as in DTH to virus.

Results

CBA mice were pretreated with ginseng total saponins to see whether such treatment will affect the cytotoxic T-cell response of the animal to a subsequent influenza virus infection. The animals were divided into three groups. The first group (2 mice) was the control receiving no treatments. The second group (3 mice) was infected with A/WSN virus. The third group (2 mice) was given 0.2 mg of ginseng total saponins per mouse (approximately 10 mg/kg body weight) 2 d before infection with the virus. All groups of mice were then tested for cytotoxicity in their spleens 6 d after the viral infection. The target cells used were P815. The results of the experiment are shown in Table 1. No significant difference ($p \geq 0.1$) was observed between the group of mice treated with ginseng (the third group) and the group untreated (the second group) in the extent of cytotoxic T-cell activity against the virus.

In another set of experiments ginseng was tested to see whether the total saponins will affect the NK activity of virus-infected mice. CBA mice were divided into five-groups. The first group (2 mice) was the control, receiving no treatments. The second group (3 mice) was given 0.2 mg of the total saponins per mouse 2 d before the NK activity assay. The third group (3 mice) was given 1.0 mg of total saponins per mouse 2 d before the assay. The fourth group (3 mice) was infected with the virus 2 d before the NK activity assay. The fifth group (3 mice) was given 0.2 mg of total saponins per mouse 4 d before the assay; the mice were then infected with the virus 2 d before the assay. The target cells used were RBL5 and YAC. The effector to target cells ratios were 25:1, 50:1 and 100:1. Typical results for the experiment are shown in Table 2. For the two target cells used, no obvious differences were observed in the NK activity between the normal group (Group 1) and the groups treated with the saponins (Groups 2 and 3), and between the virus infected group (Group 4) and the saponin-treated plus virus-infected group (Group 5).

Table 1. Effect of ginseng treatment on cytotoxic T-cell response of mice to a subsequent influenza virus infection

Treatment of mice	% specific lysis (mean ± S.E.M.)*			
	Target cells uninfected	Target cells infected		
	50:1+	25:1+	50:1+	100:1+
Control	4.8 ± 1.6	4.9 ± 0.9	12.0 ± 0.8	24.4 ± 0.8
Virus infected	5.8 ± 0.7	49.8 ± 1.1	80.2 ± 1.4	97.0 ± 2.5
Ginseng treated & virus infected	3.3 ± 1.4	53.2 ± 1.2	86.3 ± 2.8	99.1 ± 1.3

* For each experiment, no. of replicates = 4.
\+ Effector to target cells ratio.

Table 2. Effect of ginseng treatment on natural killer cell activity of mice.

Treatment of mice	% specific lysis (mean ± S.E.M.)*					
	RBL5			YAC		
	25:1+	50:1+	100:1+	25:1+	50:1+	100:1+
Control	2.7 ± 0.4	5.7 ± 0.4	12.4 ± 1.4	23.3 ± 0.4	37.4 ± 0.8	55.6 ± 1.0
0.2 mg saponins	1.9 ± 0.6	7.0 ± 0.2	12.5 ± 0.7	29.0 ± 6.6	40.3 ± 3.6	58.6 ± 2.7
1.0 mg saponins	1.8 ± 0.4	4.5 ± 0.2	9.9 ± 0.9	18.6 ± 1.0	35.9 ± 4.3	47.2 ± 1.5
Virus infected	25.9 ± 1.0	36.7 ± 1.4	53.4 ± 2.9	54.9 ± 1.8	76.5 ± 4.2	87.1 ± 2.3
0.2 mg saponins & virus infected	25.2 ± 0.6	42.8 ± 0.8	65.2 ± 1.3	54.8 ± 2.1	76.5 ± 2.6	101.7 ± 1.6

* For each experiment, no. of replicates = 4.
\+ Effector to target cells ratio.

The next set of experiments were done to see whether treatment of mice with ginseng can affect the DTH response to influenza virus infection. CBA mice were divided into three groups, each contains 3 animals. The first group was given infectious A/WSN virus on day 0, then challenged with irradiated virus 6 d later. The second group was given the total saponins (0.2 mg/mouse) 2 d before day 0 and was then infected and challenged as in the first group. The third group was treated the saponins (0.2 mg/mouse) 2 d after virus infection and was then challenged. For all the three groups, footpad swelling was measured 24 and 48 h after the challenge. Table 3 summarized the results of this experiment and it can be seen that the DTH response to the virus was suppressed in case the total saponins were given to the animals before the virus infection. If the saponins were given after the infection, no suppression was observed.

The dose dependence of the suppressive effects of ginseng total saponins was then determined. CBA mice were divided into four groups (3 mice per group). The first group was the control given infectious A/WSN virus and then challenged with UV irradiated virus. No ginseng saponins were applied. The second group was given 1.0 mg of total saponins per mouse 2 d before the viral infection. The third group was given 0.2 mg of total saponins per mouse and the fourth group was given 0.04 mg /mouse. Footpad swelling was measured 24, 48 and 72 h after the challenge. Typical results of the experiment are shown in Table 4. It can be seen that at a dose of 1.0 and 0.2 mg/mouse ginseng total saponins effectively suppressed the DTH to viral infection. The suppressive effect was still evident at a dose as low as 0.04 mg/mouse.

Table 3. The effect of ginseng treatment on DTH to viral infection

Time of ginseng administration	Increase in footpad thickness, % (mean ± S.E.M.)*	
	24 h+	48 h+
—	32.3 ± 1.1	17.7 ± 1.0
2 d before infection	7.3 ± 1.1	3.1 ± 0.0
2 d after infection	31.3 ± 0.0	18.8 ± 2.6

* For each experiment, no. of mice = 3.
+ Time after challenge.

Table 4. Dose dependence of the suppressive effect of ginseng total saponins on DTH to viral infection

Dose of saponins (mg/ mouse)	Increase in footpad thickness, % (mean ± S.E.M.)*		
	24 h +	48 h +	72 h +
0	41.7 ± 2.1	26.1 ± 1.1	14.6 ± 1.1
1.0	8.3 ± 2.1	2.1 ± 2.1	0.0 ± 0.0
0.2	9.4 ± 1.8	4.2 ± 2.1	3.1 ± 1.8
0.04	28.1 ± 1.8	14.6 ± 1.1	7.3 ± 1.1

* For each experiment, no. of mice = 3.
+ Time after challenge.

The observed suppressive effect of the saponins is not restricted to DTH to virus, but is also observed in DTH response of mice to heterologous erythrocytes-SRBC. A typical example of this is shown in Table 5. DTH to SRBC is of tuberculin type (Mitsuoka *et al.*, 1978) and total saponins of ginseng at a dosage of 1 mg/mouse injected intravenously 2 d before sensitizing the mice with SRBC suppressed DTH to subsequent challenge completely 24 and 72 h after the challenge. Suppression at 48 h was incomplete.

Table 5. Effect of ginseng on DTH to sheep erythrocytes

Treatment of mice	Increase in footpad thickness, % (mean ± S.E.M.)*		
	14 h +	48 h +	72 h +
Unsensitized, challenged.	1.6 ± 1.6	0.0 ± 0.0	0.0 ± 0.0
Sensitized, challenged.	34.4 ± 1.8	34.4 ± 1.8	19.8 ± 1.1
Ginseng treated, sensitized & challenged.	1.1 ± 1.1	14.6 ± 2.1	3.1 ± 3.1

* For each experiment, no. of mice = 3 (except in the first group, no. of mice = 2).
+ Time after challenge.

Discussion

The results of the present investigations indicate that at a dose of 0.2 mg/mouse total saponins of ginseng have no obvious effects on the Tc activity, NK activity and humoral antibody production of CBA mice responding to A/WSN influenza virus infection. On the other hand, the saponins effectively suppressed DTH to the virus at the above mentioned dosage. A lower dose of 0.04

mg/mouse still has the suppressive effect, but not as effective as the higher doses.

The anti-inflammatory action of ginseng has been described in the literature before and was found to be prophylactic (Popov & Goldwag, 1973). This agrees with the findings of the present investigations since it was observed that total saponins of ginseng is only effective in the suppression of the DTH response when administered to the animal before the viral infection, but not after.

Ginseng extracts have been well known as agents against stress (Brehkman & Dardymov, 1969 ; Popov & Goldwag, 1973) and this prophylactic effect has been suggested to be due to the active principle of ginseng functioning as steroid analogues (Bittles, Fulder, Grant & Nicholls, 1979). The total saponins of ginseng, or ginsenosides include Ro, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, Rg_3 and Rh, designated according to their migration sequence in thin-layer chromatography (TLC). Ro has the basic structure of oleanolic acid, Rb_1, Rb_2, Rc and Rd have the structure of 20S-protopanaxadiol (Sanada, Kondo, Shoji, Tanaka & Shibata, 1974a) while Re, Rf, Rg_1 and Rg_2 have the structure of 20S-protopanaxatriol (Nagai, Tanaka & Shibata, 1971; Sanada, Kondo, Shoji, Tanaka & Shibata, 1974b). The protopanaxadiols and protopanaxatriols all possess a steriod nucleus or perhydrocyclopentanophenanthrene. Since steroids, such as glucocorticoids, at supraphysiological doses are known to have anti-inflammatory and immunosupressive actions, the prophylactic DTH suppression action of ginseng total saponins observed in the present investigations may be related to their steroid-like structure.

The three different parameters tested in the present investigations involved different subpopulations of lymphoid cells, namely Tc, NK, and DTH T-cells (Td). The total ginseng saponins seem to be only active on the last category of cells responding to viral infection. The specificity, Ly phenotype and H-2 compatibility requirements of effector cells in DTH responses to influenza virus infection in mice has been investigated in details by Leung *et al.* (1980) Studies on the effects of individual ginsenosides on immune responses as well as the mechanism of the suppression of the delayed-type hypersensitivity are in progress.

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