Immunomodulatory Response Induced by Ginseng

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Abstract

There has been continuing interest in the development of synthetic and natural compounds that modify the immune response particularly for the treatment of AIDS and cancer. During the past fifty years, numerous scientific studies have been published on ginseng (Foster and Chongxi, 1992). Modern human studies have investigated preventive effect of ginseng on several kinds of cancer (Yun *et al*, 1993,Yun, 1995,Yun and Choi, 1998), its long term immunological effect on HIV patients (Sankang, 1989, Cho *et al*, 1997), its effect on cell mediated immune functions in healthy volunteers (Scaglione *et al*, 1990). Similarly non clinical studies on animal model system have studied the chemopreventive action of ginseng on cancer (Kumar, 1993,98) and immunological properties of ginseng (Kim *et al*, 1990, Tomoda *et al*, 1993, Yun *et al*, 1993, Mizuno *et al*, 1994,Lee *et al*, 1997, Park *et al*, 2001,Yoshikawa *et al*, 2001, Wang *et al*, 2001).

The precise mechanism of action of ginseng, however, not clearly understood. Considering its wide-ranging therapeutic effects, this study is being undertaken to elucidate the general mode of action of ginseng, especially to test our hypothesis that its biological action may be mediated by the immune system.

Material and Methods

In vitro techniques used for the evaluation of immunomodulatory property of plant extract:

LTT (Lymphocyte Transformation Test)

LTT is primarily based on the proliferation of lymphoid cells upon stimulation by mitogen or compound

Preparation of Spleen Cells

Swiss albino mice were killed by cervical dislocation and spleen was removed aseptically in

Table 1. Experimental design

Group	1-3	4-6	7-9	10-12
A	R+M	R+Con A (50 µl) +M(50 µl)	R+Con A(100 µl)	R+Pred (100 µl)
B	R + M	R + PHA (10 µl) +M (90 µl)	R+PHA (20 µl) +M (80 µl)	R+PHA (100µl)
C	R_1+G_1 a (100 µl)	R_1 +G_1 a (10 µl) +M (90 µl)	R_1+G_1 b(100 µl)	R_1 +G_1 b (10µl) +M (90 µl)
D	R + M	R_1 + M	R_1+Con A(100 µl)	R_1+Pred (100 µl)
E	R+ PHAP	R1 + Alcohol		

R = 100 µ spleenocyte (5×10^6 cells/ml)
R1 = 100 µl of spleenocytes containing 2 µg PHA/ml (5×10^6 cells/ml)
M = 100 µl cRPMI
G = ginseng
Con A = 100 µl/50 µl (2 µg/ml)
Pred = 100 µl of Prednisolone (100 ng/ml)
PHA = 100/200/10 µg of phytohemagglutinin (10 µg/ml)
PHAP = 100 µl phytohemagglutinin (4 µg/ml)

RPMI medium. Single cell suspension was made by teasing the tissues. Cell suspension was centrifuged at 400xg for 5 minutes. 5 ml RPMI was added and centrifuged. The pellet was shed with 2 ml of RPMI and the cells were resuspended in 2 ml RPMI. Cell density was counted with the help of a haemocytometer. Viability of the cells was checked with trypan blue and finally the suspension was maintained 5×10^6cells /ml. Then cells, medium, ginseng, mitogens and standard were added to a 96 well plate culture plate according to the protocol in Table 1.

The plate was placed in a CO_2 incubator at 37°C for 72 hrs. Each well was pulsed with 10µl of (^{3}H) thymidine (50 µCi/ml) in RPMI medium and the plate was transferred back to the incubator. After 18hrs cells were harvested using a cell harvester. They were washed thrice with PBS, once with distilled water and finally with methanol. Dried filters were dispensed into scintillation vials and 7 ml of scintillation fluid was added. Radioactivity was measured by a β liquid scintillation counter. TI (Transformation Index) was determined by the following formula

$$TI = \frac{\text{Mean Counts in Ginseng Treated}}{\text{Mean counts in control}}$$

Immunomodulatory property of a compound is confirmed by its TI value. If TI value is more than

2, the compound is considered to be an immunoistimulant, less than 0.05 is an immunosuppressor.

Macrophage Activation Test

Procedure:

Mice were killed by cervical dislocation. Abdominal wall was exposed and 5 ml of cRPMI medium containing 5μ/ml heparin was injected into the peritoneal cavity. The injected medium was circulated in the cavity by gentle massage. The medium was aspirated with the help of a syringe and peritoneal excudates cells (PEC) were collected in a siliconized test tube. After checking the viability of the cells by trypan blue and counted in a haemocytometer, a cell suspension of 2×10^6 viable cells/ml was made.

100μl of PEC was disposed into 6 rows of 96 well flat bottom culture plate. Plate was placed in a humid CO_2 incubator for 90 minutes at 37°C. Medium was removed and adherent cells were washed thrice with RPMI. 120 μl of c RPMI containing different concentration of ginseng was dispensed in each well. The plate was transferred into CO_2 incubator for 24 hrs.

Assay of Nitric Oxide (NO)

100 μl of culture soup was transferred into respective wells of another ELISA plate. 100 μl of Griess reagent was added and allowed to stand at room temperature for 20 minutes. Subsequently the absorbance was recorded in ELISA recorder at 540 nm. Standard curve of NO was prepared with $NaNO_2$ (stock 100 μm). The amount of NO formed was calculated by comparing the O.D. with standard ($NaNO_2$)

Estimation of Enzymes

After performing the NO assay TDW was added to each well.The content of the platewas subjected to three cycles of freezing and thawing to measure the Acid Phosphatatse and β-glucosonidase.

Acid Phosphatase:

Plates were subjected to a reaction mixture (containing acetate buffer, water, macrophage lysate and p-nitophenyl phosphate). Mixture was incubated at 37°C for 30 minutes. Then 40 μl of Na0H was added to stop the reaction. Optical density was read at 405 nm in Elisa recorder. Amount of product was determined from standard graph of p-nitrophenol. Enzyme activity was

expressed in nmol p-nitrophenol formed/24 hrs/mg protein.

β-Glucosonidase.

Acetate Buffer,Phenophthaline glucornic acid and enzyme macrophage lysate were mixed The contents were incubated at 37°C for 30 minutes. After this 100 µl of stop mixture (glycine, $NaCO_3$ and NaCl, NaOH, TDW) was added. Extinction was read on Elisa recorder at 540 nm. Amount of product formed was measured from standard graph of phenophthaline. Enzyme activity was expressed in nmol phenophthaline formed /24/hrs/mg protein

More amount of NO produced indicates activation of macrophages produces more enzymes. Enzymatic digestion is another killing mechanism of macrophage.

In vivo evaluation of immunomodulatory property of ginseng

Mouse sheep-RBC model

Assessment of Humoral Immunity was done by plaque forming cell assay (PFC). Principle behind the PFC is complement-mediated lysis of the red cell. When suspension of lymphoid cells from animal immunized with SRBC is incubated with SRBC. The antibodies secreted by the plasma cell cause lysis of the SRBCs in the presence of complement. The zone of lysis is called plaque. Number of plaque is a direct indication of number of antibody secreting cell.

4.5 ml of solution of agarose (1.2%) prepared in PBS was poured in petridishes. The dishes were placed on a perfectly levelled surface and allowed to solidify. The mice already treated with ginseng and SRBC, as described above, were killed by cervical dislocation. The spleen was removed, cleared of fat bodies and washed in PBS. A piece of spleen was transferred to fresh PBS and teased. The lymphatic suspension was aspirated and centrifuged at 900g × 3 min. The supernatant was discarded and the pellet was resuspended in 2 ml of PBS. Counting was done and 10^6 cells were maintained.2ml of 0.6% agarose prepared in RPMI 1640medium was added to a series of tubes. The tubes were kept at a water bath and maintained at 45°C. To these tubes 100 µl of 20% SRBC added and 10^6 lymphocyte suspension of cells were plated. The contents were gently mixed and immediately layered onto the 1.2% agrose layer prepared in advance in 35 mm petridishes. The dishes were incubated at 37°C in a humid chamber for 2-3 hours. Subsequently 1.5 ml of 10% guinea pig serum was added to each dish. The dishes were returned to the humid chamber. After 2-3 hrs when plaques became visible the serum was removed and the reverse side

(bottom) of the dishes were divided into 4 segments. Each segment was counted for the number of plaques.The mean and standard deviation was computed for each group.

HA (Haemagglutination) titer.

The blood from mice prior to killing for the PFC assay was collected from retro orbital plexus in small tubes. Tubes were kept at room temperature and were allowed to clot. Tubes were kept overnight in a refrigerator and serum was removed. The serum was cleared by centrifugation at 900gx 10min.To each well of a 96 microtitre plate 50 µl of PBS was added. 50 µl of each serum was added to the Ist well of each row diluted serially. Thus dilution in the range of 1:2,1:4,1:8,1:16,1:32, 1:64 etc till 1:4096 were obtained for each serum. One row of the plate served as control that was not supplied with serum.25 µl of 1% SRBC were added to each well. The plate was covered and left undisturbed at room temperature for 2 hrs. Each plate was then assessed for bead formation.

Assessment for Non-specific Immune response

Macrophage form bodys first defense. They kill pathogen non-specifically. When they are stimulated they show migration. This criterion is used to determine whether macrophages are stimulated or not. MMI (Macrophage Migration Index) is used as a measure to quantitate the MMI.

Procedure:

Mice were killed by cervical dislocation. The abdominal skin was opened to expose the peritoneum. 5 ml sterile RPMI medium containing heparin (10 µ/ml) was injected and the peritoneum was gently massaged. The medium was withdrawn from the peritoneal cavity and transferred into siliconized tubes. The macrophages were collected by spinning at 400g × 3 min and resuspended in 150 µl RPMI medium. Glass capillaries were filled with the cells suspension and the end was sealed with plastiscine and centrifuged at 400g × 3 min. The capillaries were cut at the interface with diamond pencil. The capillaries were fixed in a chamber filled with RPMI medium. Migration chamber was kept at 37°C. Migration was drawn on filter paper by microscope. Migration area was cut and weighed. MMI was calculated by the following formula:

$$\text{MMI} = \frac{\text{Migration area for test compound}}{\text{Migration area for control}}$$

MMI value more than 1 indicates stimulation and less than 1 means suppression of the macrophage activity.

Assessment of Cell Mediated Immunity

For the purpose of assessing cell mediated immunity, mouse foot assay is used as a criterion (to measure DTH). This type of lymphocyte involvement in CMI is T- lymphocyte.

Procedure

Mice previously immunized with SRBC were injected with 50% SRBC in right foot and in left foot pad with PBS. After 24-hrs thickness of both footpads was measured by schnell tester.

The difference between two was used as an index of DTH reaction

Results

Results are presented in Tables 2-5

Table 2 shows the immunomodulatory effect of ginseng. Con A and PHA were used as a positive controls and prednisolone as negative control. TI values more than 2 were considered to be

Table 2. *In vitro* evaluation of immunomodulatory property of ginseng (by LTT)

Compound	Concentrations	Mean DPM counts	TI
Control		1068	1
Concavaline A	0.5 μg	286586	268.33
	1 μg	264448	247.61
Prednisolone	50 ng	673	0.63
Phytohemagglutin	0.5 μg	5652	5.29
Phytohemagglutin	1.0 μg	6632	6.20
Phytohemagglutin	5.0 μg	28462	26.64
Ginseng	0.01 μg	2978	2.78
Ginseng	0.1 μg	3268	3.05
Ginseng	1.0 μg	3632	3.40
Ginseng	10 μg	4854	4.54
PHAP	2.5 μg	2452	2.29
Alcohol		762	0.71

Table 3. Effect of Lipopolysachharide(LPS)/Ginseng on generation of NO by mouse Macrophage

Group	Control Nil	n moles of NO generated /24 hrs/ 10^6 PEC		
		1 μg	0.5 μg	0.1 μg
Control	4.38 ± 1.13	–	–	–
LPS	–	17.62 ± 1.16	17.64 ± 1.62	18.32 ± 2.31
Ginseng	–	20.4 ± 2.24	16.16 ± 1.24	18.45 ± 1.64

Values are expressed as means of ± SD of 3 experiments, each performed in triplicate

Table 4. Effect of ginseng on activities of β-glucosonidase and acid phosphatase Enzymes in mouse macrophage.

Seria	Compound	β-glucosonidase (*n mol/24 hrs/ mg protein*)			Acid Phosphatase (*n mol/24 hrs/ mg protein*)		
		Nil	1 μg	0.5 μg	Nil	1 μg	0.5 μg
1	Control	36.30 ± 3.42	–	–	76.32 ± 2.38	–	–
2	Ginseng		165.42 ± 8.46	204.64 ± 12.64		265.0 ± 7.4	297.62 ± 16.72

Values are expressed as means of ± SD of 3 experiments, each performed in triplicate

Table 5. Effect of Ginseng on Immune Responses of Mouse

Parameter of Immune Response	Untreated	Ginseng treated
HA Titer	136 ± 0.06	1546 ± 124
Plaque forming cells/10^5spleen cells	67.5 ± 5.38	264 ± 18
DTH response (mm)	0.56 ± 06	1.24 ± .04
Macrophage Migration Index (MMI)	1.0 ± .02	4.52 ± .06

immunomodulatory stimulant and TI valuing less than 2 were taken as immunosuppressive. Four different concentration of ginseng (0.01,0.1,1,10 μg) was tested for TI value. TI values for different concentrations of ginseng were dose dependent. The TI value was more than 2 at 1 and 10 μg concentration. Thus it shows that ginseng stimulate spleenocytes *in vitro,* they are potent immuno stimulant.

Table 3 shows different concentrations of ginseng tested to see their effect on NO generation by PEC of mouse. Ginseng treated macrophages showed significant increase in NO production as compared to cell control (untreated macrophage)

Table 4 shows that ginseng enhanced the activity of both enzymes: Acid Phosphatase and β-glucosonidase.

Table 5 shows the effect of ginseng on humoral and cell mediated immunity (CMI). HA titer approximately 10 fold increases and there was 1.7 fold increases in the PFC count. Thus it increases the humoral response significantly. Enhancement of CMI was determined by DTH response. It was found to be 3 folds.

The evidence regarding the enhancement of non-specific immune response by ginseng is provided by data on macrophage activation. Ginseng treated macrophage showed approximately 3-fold increase in Macrophage Migration Index (MMI). Activation of macrophage is of great significance because macrophages are Ist line of defense.

Discussion

The results of the present study demonstrate that ginseng has immunomodulatory effects and its action primarily mediated by macrophages. The initial response of ginseng appears to have chemotactic stimulus for macrophages. The macrophages having acquired these functional attributes following ginseng treatment are likely to be more efficient in antigen uptake, processessing and presentation to lymphocytes during subsequent antigenic challenge. The ginseng treatment also showed to increase the NO secretion within the macrophage thereby generating a powerful anti microbial system. More amount of NO produced indicates activation of macrophages. Similarly activated macrophages produce more enzymes, thus inducing efficient killing mechanism of macrophages.

The proliferated spleenocytes also activates the NK cells *in vitro* through secretion of endogenous IL-2. This kills the tumor target cells more efficiently (Yun *et al*, 1993) This study in fact has shown that ginseng treatment significantly enhances the *in vitro* lymphocyte proliferative response of spleen cells. There is an enhanced increase in the T helper and T cytotoxic cells (Yamada *et al*, 1994) It seems that ginseng acts as an immunostimulant that facilitates or precipitate an interaction between various immune cell populations resulting in an activated immune status that responds more dynamically to subsequent antigenic exposure. In the present study it was observed that the ginseng stimulates the activity of antibody secreting cells. Thus enhancing the mitogenesis of T and B-lymphocytes.

The present study suggests that the immunomodulatory properties of ginseng could be used for general prophylactic purposes and also in situations demanding the stimulation of cellular immune response mechanisms to react more efficiently against a wide range of pathogens or tumors.

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