# IMMUNOMODULATORY ACTIVITY OF *PANAX GINSENG* EXTRACT

**V.K. Singh*, S.S. Agarwal** and B.M. Gupta***

** Division of Virology, Central Drug Research Institute, Lucknow-226 001, India.*

*** Department of Medicine, King George's Medical College, Lucknow-226 003, India.*

## ABSTRACT

*Panax ginseng* extract is capable of enhancing antibody plaque forming cell response and circulating antibody titre against sheep erythrocytes, cell mediated immunity against Semliki forest virus antigen and natural killer cell activity in mice. It also enhanced production of interferon of both the pH stable and labile types induced by an interferon inducer of fungal origin.

## INTRODUCTION

*Panax ginseng* C.A. Meyer (Araliaceae) is one of the Chinese drugs which is said to be a tonic or a drug of longevity. It has been used as an important folk drug for treatment of various diseases including diabetes mellitus in many Asian countries, such as China, Korea, Japan and Russia. On the basis of pharmacological properties, ginseng extract has been classified under a new class of compounds known as adaptogens or antistress agents. According to Breakhman and Dardymov* an adaptogen should meet the following criterion: (a) It should be innocuous and cause minimal disorders in the physiological functions of an organism (b) The action of an adaptogen should be nonspecific, and (c) An adaptogen may possess normalizing action irrespective of the direction of the foregoing pathologic change. The important pharmacological activities of *P. ginseng* extract include (a) enhancement of swimming time (b) prevention of stress induced pepticulcers (c) proliferation of endoplasmic reticulum and ribosomes in rat hepatocytes (d) inhibition of glycogen utilisation in rat skeletal muscle during exercise and (e) inhibition of adrenaline induced lipolysis in fat cells.[2-7] When administered orally, in combination with 6-MFA (an interferon inducing antiviral substance of fungal origin), it significantly enhances the protection of mice against Semliki forest virus (SFV) compared to 6-MFA alone.[8] The mechanism of this synergistic effect by ginseng is not fully understood. *In-vitro* studies on human peripheral blood lymphocytes have shown that besides inducing interferon (IFN), ginseng extract augments natural killer (NK) cell and antibody dependent cytotoxic (ADCC) activities.[9] This has led us to evaluate the effect of ginseng extract on the expression of humoral and cell mediated immune responses in mice to sheep red blood cells (SRBC) and SFV antigens. The results are reported in this paper.

## MATERIAL AND METHODS

*Panax ginseng extract*: Ginseng extract (G 115 lot 19269) was the generous gift from Pharmaton SA, Lugano-Bioggio, Switzerland. One gm

of the extract (powder) corresponds to 5 gm of root. It was found to be safe upto 30 gm/Kg in mice when administered orally in a single dose. Ginseng was given orally at dose of 10 mg/mouse/day for four consecutive days for studying its immunoregulatory properties.

*6-MFA:* It is an IFN inducing antiviral substance that was prepared from cultures of *Aspergillus ochraceus* ATCC 28706 as described previously.[10] Chemically 6-MFA is a polysaccharide nucleoprotein complex containing mycovirus particles with doublestranded RNA (dsRNA). Animals were given preparations of 6-MFA intraperitoneally at dose of 3 mg/mouse. This dose of 6-MFA has been shown to protect significant proportion of mice against SFV.[11]

*Mice:* Thirty five day old Swiss albino mice bred randomly at CDRI, weighing 14-15 gm, were used for all experiments. The animals were kept with unlimited access to water and standard mouse diet (Hindustan Lever Ltd., Bombay).

*SFV:* It was originally obtained from ATCC[12] and was maintained in our laboratory by intracerebral passages in CDRI-Swiss outbred mice. It has undergone seventy three passages in our laboratory.

*Antibody plaque forming cell response:* Antibody forming cells in spleens of test mice (five per group) were enumerated employing the localised agar gel haemolytic technique of Jorne and Nordian[13] with slight modifications[14] in which concentration of splenocytes and SRBC varied. Agarose was used in place of agar. After initial incubation of spleen cells and SRBC in agarose for 2 hr at 37°C the test plates were flooded with complement (fresh guinea pig serum diluted 1:10 with MEM) and further incubated for 90 min. at 37°C. Antibody plaques were counted and recorded.

*Haemagglutinating antibody assay:* Mice (five per group) receiving ginseng extract before SRBC were bled on different days. Sera samples of all animals in treated (ginseng) were pooled, so also in the control and inactivated at 56°C for 30 min. Two fold dilutions of sera samples were made in phosphate buffer saline (PBS, pH 7.2) in microtitre Plates and mixed (1:1) with one percent suspension of SRBC in PBS. End points of agglutination were noted after incubation for 90 min. at room temperature (30°C).

**Table 1.** Haemagglutinating antibody and plaque forming cell responses to SRBC antigen in mice pretreated with ginseng extract.

| Batches of SRBC* | Treatment of mice with (before SRBC administration) | Serum** HA titre (4th day) | Fold rise in HA titre | Plaque forming cells*** per million splenocytes |
|---|---|---|---|---|
| I | (a) PBS (Control) | 32 | 2 | 2,215 |
| | (b) Ginseng | 64 | | 2,487 |
| II | (a) PBS (Control) | 128 | 8 | 272 |
| | (b) Ginseng | 1,024 | | 776 |
| III | (a) PBS (Control) | 1,024 | 2 | – |
| | (b) Ginseng | 2,048 | | – |

* SRBC batches differed in source and storage life.
** Pooled from five mice on the 4th day post SRBC.
*** Splenocytes pooled from five mice.
– Not done.

*Migration inhibition test*: The test was carried out according to method described by David et al.[15] Spleen cells were taken from mice that had been sensitised with SFV and were used as source of macrophage. SFV antigen (sucrose acetone extract) was prepared from infected brain tissue as described by Clarke and Casals.[16] The control chamber of test plates contained normal brain extract in place of the antigen. Duplicate sets of chambers were prepared for antigen and the appropriate control. All chambers were closed with cover slips and sealed with paraffin wax. The plates were then incubated for 18-24 hr at 37°C. Area of migration was drawn on the graph paper and percent migration inhibition was calculated.[17]

Percent migration =

$$\frac{\text{Mean area with test antigen}}{\text{Mean area with normal brain extract}} \times 100$$

Percent migration inhibition = 100 - percent migration.

*Assay of NK cell activity*: The test was carried out as described by Engler et al[18] using chick erythrocytes (CRBC) as target cells in place of YAC – 1 tumor cells. Target cells were incubated at 37°C for 90 min. with $^{51}Cr$ (1.0 mCi/ml, specific activity 64 mCi/mg, Bhabha Atomic Research Center, Bombay), at a concentration of 150/µl of $^{51}Cr$ per $3 \times 10^7$ cells. After 90 min. target cells were washed three times with RPMI 1640 and adjusted to a concentration of $10^5$ cells/ml. Concentration of splenocytes, taken from animals on the 7th day post ginseng administration, was adjusted to $10^6$ cells/ml. Equal volumes (500/µl each) of splenocytes and target cells were mixed (cell ratio 10:1) in tubes and incubated for 4 hr at 37°C. For low (spontaneous) control release, the untreated target cells were incubated with RPMI 1640, for high control release the targets were lysed by treatment with 1.0 percent Triton X-100. Release of $^{51}Cr$ into the supernatant was measured in scintillation counter and the specific $^{51}Cr$ release was calculated according to following formula:

Percent specific lysis =

$$\frac{\text{Test release - low control release}}{\text{High control release - low control release}}$$

*Induction of IFN and its assay*: Two groups of animals, comprising 20 each, were taken for this study. One group received 6-MFA (3 mg/mouse) intraperitoneally. The second group received ginseng extract 10 mg/mouse/day for

**Table 2.** Haemagglutinating antibody response to SRBC antigen in mice pretreated with ginseng extract assessed at intervals.

| Batches of SRBC | Pretreatment | HA titre* (days post SRBC inoculation) 4 | 10 | 20 |
|---|---|---|---|---|
| I | (a) PBS (Control) | 32 | 64 | 128 |
| | (b) Ginseng | 64 (2) | 256 (4) | 1,024 (8) |
| II | (a) PBS (Control) | 32 | 128 | 128 |
| | (b) Ginseng | 64 (2) | 512 (4) | 1,024 (8) |
| III | (a) PBS (Control) | 32 | 128 | 256 |
| | (b) Ginseng | 64 (2) | 512 (4) | 1,024 (4) |

* Sera samples pooled from five animals.
The figure in brackets denote fold increase in HA titre due to ginseng extract.

Table 3. Macrophage migration inhibition (%) of sensitized mice on different days post antigen injection.

| Treatment of mice with | Percent migration inhibition on different days post challenge | | | | | | |
|---|---|---|---|---|---|---|---|
| | 2 | 3 | 5 | 7 | 10 | 16 | 20 |
| (a) Control (Antigen alone) | 43 | 21 | 10 | 3 | 0 | 2 | 0 |
| (b) Treated (Ginseng + antigen) | 5 | 2 | 0 | 10 | 48 | 71 | 72 |

Splenocytes from five mice were pooled at each of the seven time intervals.

4 days and on the 5th day received 6-MFA as before. Animals from both groups were bled at intervals of 6 and 18 hr. following 6-MFA injection. Serum samples from each group were pooled separately and were lyophilised. Normal mouse serum was processed similarly and designated "mock" IFM. Before use, lyophilised sera samples were reconstituted to their original volume by addition of sterile distilled water. Test as well as the mock IFN samples were divided into two equal portions. One portion was incubated with tris-HCl (0.2 molar) pH2 for 48 hr. at 4°C, and the other portion left untreated. All treated samples were brought back to pH7 by addition of sodium hydroxide before final titration.

IFN assay was carried out. in mouse LM cells ($L_{929}$) using Vesicular stomatitis virus (VSV) as challenge virus in multicavity polysterene plate (Laxbro, Pune) and was based on methods of Wagner.[19] The cell sheets were challenged with VSV (0.1 ml volume containing 30-40 PFU/well). After challenge, the plates were further incubated at 37°C in $CO_2$ incubator for a period of 1 hr. and the wells were overlaid with 0.5% carboxymethyl cellulose (Sigma). Plates were again incubated for 44-48 hr. and observed for the appearance of plaques. Standard mouse IFN (No. G002-904-511, obtained from Institute of Infectious Disease, NIH, Bethesda, Maryland, USA) and the mock IFN served as controls. One unit of IFN was ascertained as the amount present in the highest dilution which reduced the plaque counts down to 50% of untreated controls. One unit of IFN in the present system was equal to appromimately three international units (i.u.).

Table 4. Natural killer cell activity* in mice treated with ginseng extract.

| Expt. No. | Treatment of mice with | Percent specific lysis |
|---|---|---|
| I | (a) PBS (Control) | 52 |
| | (b) Ginseng | 92** |
| II | (a) PBS (Control) | 52 |
| | (b) Ginseng | 82** |

* Splenocytes from five animals were pooled in each group.
** 'P' value < .01, paired t test.[25]

## RESULTS AND DISCUSSION

The results of haemagglutinating antibody (HA) titre and number of plaque forming cells in three separate experiments performed with different batches of SRBC as antigen, are presented in table 1. Data show that the number of antibody forming cells, as well as the titres of circulating antibodies are enhanced if test animals are pretreated with ginseng extract. The fact that enhancement took place in all the three tests under conditions of differing antigenic stimuli suggests that enhancement may be significant.

The experiment was repeated and the results presented in table 2 show humoral antibody response of ginseng treated mice to SRBC assessed at varying intervals. On fourth day after

SRBC injection, HA titre of ginseng treated group was 2 fold higher than the control group (PBS treated). On tenth day, the treated group showed 4 fold higher HA titre, and on 20th day the response in the ginseng treated group was 4-8 fold higher than the controls. Here, as in Table 1, enhancement took place in all three tests with differing antigenic stimuli, suggesting that enhancement may be significant.

Results of inhibition of splenocytes (macrophages) migration (a parameter of cell mediated immune response, CMI) measured at different times post SFV antigen exposure are presented in Table 3. In control animals, the migration inhibition response was initially at a higher level which declined with time. In animals pretreated with ginseng response was reversed. The response was initially at a lower level but reached higher peak at a later period. These results show that ginseng treatment modulates the CMI response in test animals.

Results of two separate experiments on natural killer cell activity are presented in Table 4. Data show that ginseng extract significantly elevated the NK cell activity in comparison to control animals.

The effect of pretreatment with ginseng extract on IFN response is shown in Table 5. Data show that the total amount of IFM induced at 6 and 18 hr. after the administration of 6-MFA was 2 and 8 fold greater, respectively, compared to animals given 6-MFA alone. In addition, ginseng pretreatment altered the proportions of pH2 labile and stable IFNs. At 6 hr., the proportion of pH labile to pH stable IFN in the combined treated animals was 7:1 as compared to 1:1 in animals with 6-MFA alone. At 18 hr., however, the proportion of labile to stable IFN in both groups of animals remained the same (1:2).

The results of these *in-vivo* studies reported in this paper are in agreement with our previous results of *in-vitro* studies.[9]

The synergistic effect of ginseng with 6-MFA, an IFN inducer, against SFV in mice, as reported earlier[8] may be mediated by more than one mechanism(s). Besides, enhanced production of IFN (particularly pH2 labile IFN) in association with 6-FMA (Table 5), ginseng extract by itself enhanced humoral as well as CMI responses (Table 1-4). The latter is more likely to be the mechanism of synergistic effect of ginseng. There are several reports of substances (homopolynucleotides and endotoxin) which are able to protect animals without inducing IFN.[20-22] There are also reports that immunomodulators, e.g. crude bacterial extracts, cimetidine, cyclomumine and isoprinosine, act in concert with IFN to produce enhanced antiviral activity.[23-24] The exact mechanism(s) of enhancement of antiviral resistance is not known in most cases. Ginseng appears to be an example of similar kind of immunostimulator.

**Table 5.** Interferon response (total = pH2 labile + pH2 stable) of mice given combined treatment(ginseng followed by 6-MFA) compared to 6-MFA alone, at 6 and 18 hr. after 6-MFA administration.

| | | Interferon titre (i.u./ml of serum) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Group | Treatment | 6 hr. | | | | 18 hr. | | | |
| | | Total IFN | pH2 stable (S) | pH2 labile (L) | Ratio L : S | Total IFN | pH2 stable (S) | pH2 labile (L) | Ratio L : S |
| 1. | 6-MFA | 2783* | 1397 | 1386 | 1 : 1 | 641* | 436 | 205 | 1 : 2 |
| 2. | Ginseng + 6-MFA | 5454* | 684 | 4770 | 7 : 1 | 4914* | 3481 | 1433 | 1 : 2 |

* Serum samples from 20 mice were pooled before titration for both the groups, at 6 hr. total IFN increase due to ginseng is two fold, at 18 hr. the increase is 8 fold.

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**Chong:** On the last column you flashed the side so quickly, I wasn't quite sure. Most of the mice died before day 20. Am I right? Survival rate was taken on day 20, but as I couldn't remember that slide you showed, most of the mice died between 8th day to about 14th day. Could you explain that?

**Singh:** We infected mice with Semliki forest virus and incubation period of that virus was 72 hours. Those mice start dying after 72 hours and they died after 9th day in control group. In treated group, mice also start dying as the control group. In ginseng treated group, nearly 60 to 70% of animals died between 3rd and 9th day. So MSD will be nearly 7 or 8th. In MFA treated animals, mice start dying after 9th day. That is – in control group all animals died and in 6-MFA plus ginseng treated group, you must have seen that, MSD is more than 10 days.

**Chong:** Yes, but I still don't quite understand. Could I just make one point. Are you saying that the ginseng is an antiviral substance and at the same time has an effect on B-cell lymphocyte?

**Singh:** Yes.

**Chong:** Are you saying that ginseng is effective both antivirally as well as in producing immunoglobulins? Were you able to measure the immunoglobulin in the serum?

**Singh:** No, we measured circulating antibody against SRBO sheep's red blood cells and found ginseng treatment inhanced circulating antibody.

**Chong:** Your results are astounding. They are amazing results. I think this experiment is pretty worth reapting.

**Singth:** We measured the circulating antibody three times and repeated cell-mediated immunity experiments so many times.

# 인삼추출물의 면역조절 및 항바이러스 활성 연구

V. K. Singh, S. S. Angarwal and B. M. Gupta
Division of Virology, Central Drug Research Institute, Lucknow , India

인삼은 외적 유해인자에 대한 비특이적 생체 저항력을 증진시켜주며 물리적, 화학적, 생물학적인 외적 변화에 대해 adaptogen으로서 작용한다. 인삼은 말초혈액 림파구에 의한 면역 인터페론 합성을 촉진시키며, 바이러스에 감염된 흰쥐에 대해 치료효과가 뚜렷하다. 인삼을 Semliki forest virus (SFV) 감염 전후 계속 경구투여하면 34~40%의 보호 효과가 있었다. 인삼투여로 세포의 항체생성능이 증가되었으며, sheep의 적혈구 세포에 대한 혈행항체의 역가가 증진되었다. 또한 SFV 항원에 대한 면역성도 높아졌으며, N.K. 세포의 활성도도 크게 증가되었다.

Aspergillus Ochraceus ATCC 28706 균주에서 추출한 6-MFA는 인터페론 유도물질(inducer)로서 잘 알려져 있으며 두 종류의 인터페론, 즉 산성 용액에서 안정한 인터페론과 불안정한 인터페론을 유도시킨다. 인삼은 이들 두 종류의 인터페론 생성을 모두 증진시킨다. 동물실험 결과 6-MFA를 단독 처리하면 SFV 감염에 의해 낮은 보호작용을 보이나, 인삼투여를 병용하면 SFV 감염을 100% 완전히 방지할 수 있었다.

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