EFFECT OF KOREAN RED GINSENG ON IMMUNOLOGICL MARKERS OF PERSONS WITH HUMAN IMMUNODEFICIENCY VIRUS

Y.O. Shin, Y.K. Cho*, M.K. Ki, J.S. Lee, J.G. Nam, M.H. Choi, Y.B. Kim, K.W. Choi**

Center for AIDS Research, National Institute of Health
** Medical College, Ulsan University*
***Medical College, Seoul National University*

INTRODUCTION

The numbers of persons with AIDS worldwide become 718,894 as of Jun, 30, 1993, according to Weekly Epidemiological Records published by World Health Organization, which is in charge of AIDS case report every 6 months. Of those numbers, America reported 371,086 persons with AIDS. However, the real numbers of persons with AIDS throughout the world are estimated to be 2.5 million because of underreporting, delayed report and no report in many countries. In addition, it is estimated that 13 million persons are presently infected with HIV worldwide. These numbers are consistently increasing in many areas.

So far, 276 persons with AIDS/HIV have been found since the first AIDS case in 1985 in Korea. Most of them are infected through sexual contact. The impact of HIV infection on the persons concerned are very great because most infected persons are at the age of 20s to 40s which are very active economically and socially. HIV infected persons developed died of AIDS mostly because no effective treatment agents are presently available.

AZT, ddI and ddC are licensed as the agent delaying the progress of disease in AIDS patients in the United States. However, they have many side effects and are highly expensive. Furthermore, those drugs have limited efficacy for persons with AIDS. Therefore, the development of more effective and less toxic drugs or vaccines are urgent for the treatment of patients or prevention of further infection.

Researchers in many countries competitively tried to develop more effective drugs or vaccines. AZT developed by Wellcome are known to be very effective for increasing CD4+ cells for first 6 months after taking drugs. However, thereafter, CD4+ cells show sharp declining tendency. Therefore, many attempts have been tried to slow down the speed of CD4+ cell decline by development of more advanced drugs or new treatment method.

Recently, researches on the development on anti-tat protein, sCD4 proteins and anti-enzymes of HIV in addition to nucleoside analogues are begun in many laboratories. Korean red ginseng is traditionally known to be effective for the aging, anti-cancer activity and decrease of blood sugar in Herb Medicine. Some of these facts are going to be confirmed by scientists concerned. Presently, effect of Korean red ginseng on the persons with HIV was studied by monitoring CD4+ cells, β_2-microglobulin and p-24 antigen in plasma. Anti-HIV activity by Korean red ginseng was tested on MT-4 cells by ELISA.

MATERIALS AND METHODS

Study population : A total of 193 HIV infected persons confirmed by National Institute of Health are divided into 5 groups depending on taking AZT or Korean red ginseng. AZT and Korean red ginseng were simultaneously to I-A. Korean red ginseng was given to group I-B after taking AZT. AZT only was given to group II and Korean red ginseng only to group III. None was given to groups IV, which was considered as negative control. Three hundred mg of AZT and/or 5.4g of Korean red ginseng per day were orally given to the infected persons during the research period. Numbers of each group are summarized in Table 1.

Table 1. Distribution of 193 HIV infected persons included in the study by Korean red ginseng and AZT treatment

RG	AZT	Test No.	Subtotal	Total
Taken	Treated	56	96	193
	Not treated	40		
Not taken	Treated	39	97	
	Not treated	58		

Individuals included in study are bled for testing every 3 months and control groups every 6 months.

Blood tests : Blood was taken from target population with vacutainer containing EDTA and tested within 24 hours using Coulter counter. Whole white blood cells, red blood cells, lymphocytes and haemoglobin content etc. were measured.

Measurement of lymphocyte subpopulation : 100μl of whole blood cells are reacted with Leu-3a(CD4+) labelled with fluorescein isocyanate and Leu-2a(CD8+)monoclonal antibody labelled with phycoerythrin for 30 minutes at room temperature. The results were read using flow cytometry. Average counting of cells per tests is approximately 10,000.

Measurement of β_2-microglobulin : β_2-microglobulin in plasma or sera from persons with HIV was measured by radioimmunoassay(RIA) using conjugates(Pharmacia Diagnostics AB, Sweden) labelled with I^{125}. The procedure was followed by the manual of conjugate manufacturer. β_2-microglobulin in specimens was detected by competitive inhibition method. Experimental errors among individuals were minimized by testing 40 specimens simultaneously.

Detection of p24 antigen : P24 antigen of HIV-1 virus in plasma or sera from persons with HIV was detected by enzyme linked immunosorbent assay(ELISA) using Dupont(USA) reagent. The concentration of p24 antigen was measured by standard linear graphs. P24 antigen in the supernatants from HIV infected MT-4 cell culture was also measured by antigen capture ELISA method using the Dupont reagent.

MT-4 cell culture : MT-4 cells containing HTLV-I were cultured with RPMI-1640 media composed of 10% fetal bovine serum(FBS), 100unit/ml penicillin, 100μg/ml streptomycin and 2mM/ml L-glutamine at 37℃ in 5% CO_2 incubator. Cell viability of MT-4 cells was confirmed by trypan blue dye exclusion methed. Cells were passaged once every 3 or 4 days to maintain 2.0-3.0 × 10^5cells/ml.

Preparation of HIV-1 virus : HIV-1 virus was obtained from the supernatants of H9/HTLV-III cell culture and kept -70℃ until use. Virus potency was titrated by obtaining $TCID_{50}$.

Evaluation of anti-HIV activity by compounds : Different dilution of candidate drugs were distributed into MT-4 cell culture with 500 $TCID_{50}$ HIV-1 virus/ml. 0.2ml of the suspended solution was dispensed into 96 well microplate. The microplate was maintained for 6 days at 37℃ in humidified, 5% CO_2 incubator. Antivitral activities were evaluated by MTT and antigen capture ELISA method.

3-(4, 5-dimethylthiazol-2-1)-2, 5-diphenyl tetrazolium bromide(MTT) assay : MTT assay was perfomed with commercial kit(MTT, Boehringer mannheim) using MT-4 cells. The procedures are followed by the manual of the reagent manufacturer.

Statistical analysis of surrogate markers : Linear regression model was used to obtain slope coefficients of surrogate markers among persons with HIV by time after taking Korean red ginseng/AZT.

$$y = \alpha + \beta x$$

Independent variable(x) is the period taking RG or AZT and depend variables(y) are munbers or percentage of CD4+ cell, β_2-microglobulin and p-24 antigen. α represents CD4+ count at the entry period.

Numerical values for α and β were obtained by following formula :

$$\beta = \frac{n\sum xy - (\sum x)(\sum y)}{n\sum x^2 - (\sum x)^2}$$

$$\alpha = \frac{\sum y - b\sum x}{n}$$

After making linear regression formulas for individual, the means out of slopes(β) for compairing with the difference of groups were calculated.

Statistical Analysis System(SAS) and dbaseIII$^+$ were used for all statistical analysis.

RESULTS AND DISCUSSION

Anti-HIV replication by Korean red ginseng *in vitro* MT-4 cell

Five fractions of Korean red ginseng including saponin prepared by Korea ginseng and Tobacco Research Institute were tested for inhibition activity *in vitro* MT-4 cell by MTT and antigen capture ELISA method. The results of anti-HIV activity by fractions are shown in Table 2.

Table 2. Inhibition of HIV replication by fractions of Korean red ginseng in MT-4 cell culture

Fraction No.	ED_{50} (μg/ml)	CD_{50} (μg/ml)	Selectivity Index
1	None	22.0	-
2	None	72.2	-
3	None	4.5	-
4	None	1.9	-
5	None	1,118.5	-
6	None	4,835.8	-
7	169.5	1,352.8	8.0
AZT(μM)	0.00032	5.2	16,387.1
Dextran sulphate	2.5	2,433.9	984.8

ED_{50}(50% effective dose) : Dose required to achieve 50% protection of the cells aganist the cytopathic effect of HIV(based on all viability). CD_{50}(50% cytotoxic dose) : Dose required to reduce the number of viable uninfected cells by 50%. SI : Ratio of CD_{50} to ED_{50}. None : no inhibition at the concentration of less than that of CD_{50}. - : less than one.

Fractions 1 to 6 did not show any inhibitory activity at the concentration of less than CD_{50} by MTT assay. On the contrary, ED_{50} of fraction 7 were 169.5 and 1352.8μg/ml respectively with Selectivity Index(SI) of 8.0. In fraction 3 of Fig. 1, formazan production of HIV uninfected cell control decreased rapidly at the concentration of 1 to 10μg/ml (CD_{50} : 4.47μg/ml) by dilution, whereas there were no formazan productions of more than 50% in the concentration of less than 1μg/ml in HIV infected MT-4 cells. On the other hand, in the fraction 7 of Fig. 2, there were formazan productions of more than 50 percent in the concentration of 200 to 600μg/ml with the ED_{50} of 240.95μg/ml. The highest percentage of formazan production was formed at the around 300μg/ml of fraction 7 repeatedly. The anti-HIV activity by fraction 7 was also confirmed by antigen capture ELISA, immunofluorescent antibody(IFA) tests and cytopathic effect observations.

Table 3 shows that no cytopathic effect by HIV was observed at the concentration of more than 500μg/ml of fraction 7.

There were significant inhibition of HIV antigens by IFA test in slide coated with aceton treated cell suspension where fraction 7 was maintained with MT-4 cells inoculated with HIV. However, there were no complete inhibition of HIV antigen expression from 6 days of culture. Uncomplete inhibitions of viral expression were also shown the tests with AZT.

Table 3. Inhibition of CPE by fraction 7 on HIV replication in MT-4 cells

Concentration	CPE					
(μg/ml)	Days	2	3	4	5	6
100	—	—	++	+++	+++	
200	—	—	++	+++	+++	
300	—	—	—	++	+++	
400	—	—	—	+	++	
500	—	—	—	—	+	
600	—	—	—	—	—	
700	—	—	—	—	—	
800	—	—	—	—	—	
900	—	—	—	—	—	
1000	—	—	—	—	—	
Cell control	—	—	—	—	—	
Virus control	—	—	++	+++	+++	

Cytopathic effect of drug treated MT-4 cells with HIV infection was observed by inverted microscope. —, +, ++, and +++ denote relative intensities of the cytopathic effect.

Table 4. Inhibition of HIV antigen expression by fraction 7 on HIV replication in MT-4 cells

Concentration	IFA					
(μg/ml)	Days	2	3	4	5	6
100	+	++	+++	+++	+++	
200	±	++	+++	+++	+++	
300	—	+	++	+++	+++	
400	—	—	+	++	++	
500	—	—	±	+	+	
600	—	—	—	—	+	
700	—	—	—	—	+	
800	—	—	—	—	+	
900	—	—	—	—	+	
1000	—	—	—	—	+	
Cell control	—	—	—	—	±	
Virus control	+	++	+++	+++	+++	

Viral antigen expression was examined by immunofluorescence assay. —, ±, +, ++, and +++ denote relative intensities of the antigen expression.

Fig. 3 shows that HIV antigen detected by ELISA in the supernatants of HIV infected MT-4 cells was inhibited by the degree of Korean red ginseng concentration from 3 to 4 days after culture. Similarly, it is shown that HIV antigen was decreased by increasing concentrations of Korean red ginseng 4 days after inoculation. Based on these expeimental data obtained, we can conclude that Korean red ginseng inhibits some degree of anti-HIV expression.

Clinical observations on study population

Differences of disease frequencies among groups during the study period could not be obtained because most study population had relatively high level of CD4+ cells at study entry and short study period. Generally group 2 and 4 complained of exhaustions more frequently than group not taking Korean red ginseng. It is presumed that this might be caused by psychological influence by group taking Korean red ginseng. Group I and II showed 2 AIDS cases each. Numbers of target populations by groups and death during the study period are shown in Table 5.

Table 5. Disseases among HIV infected persons by AZT and Korean Red Ginseng taking

Groups	Numbers tested	No. of Death		
		AIDS	Other Diseases	Total
I	56	2	—	2
II	39	2	2	4
III	40	—	—	—
IV	58	—	4	4

Change of CD4+ cells by months after taking compounds

CD4+ cells were measured every 3 months after taking Korean red ginseng/AZT using fluorescent activated cell sorter. Average number of CD4+ cells by group at study entry are shown in Table 6. Average numbers of CD4+ cells in AZT groups were 318.7 to 344.6, whereas those of other groups were 565.7 to 673.2.

Table 6. Mean CD4+ cells of HIV infected persons by groups at the time of taking Korean red ginseng/AZT

Groups	Obsrved No.	Mean	Minimum	Maximum
I-A	14	289.0± 129.1	135.0	581.0
I-B	31	391.4± 292.48	76.0	1,632.0
II	39	318.7± 168.4	45.0	987.0
III	40	565.7± 256.7	170.0	1,188.0
IV	50	673.2± 360.4	142.0	1,699.0

I-A : Group taking AZT and Korean red ginseng simultaneously. I-B : Group taking Korean red ginseng after AZT. II : Group taking AZT only. III : Group taking Korean red ginseng only. IV : Control group taking none.

Differences of CD4+ cells at study entry depending on AZT taking should be noted in the evaluation of efficacy of AZT and Korean red ginseng.

Change of $CD4^+$ cells 6 months after taking compounds

Means of slope coefficients of CD4+ cells among groups were obtained at entry and 6 months later as shown in Table 7, slope coefficents of CD4+ cells before study were more rapid in AZT taking group than in other groups mainly because

of low CD4+ cells in AZT groups. Six months after taking korean red ginseng and/or AZT, differences of slope coefficients among five groups were shown in Table 7. Differences of slope coefficients between 2 periods were the greatest in group I - A taking AZT and Korean red ginseng simultaneously. Slope coefficients of 4 groups except group IV were converted to plus which means increase of CD4+ cells after taking AZT or Korean red ginseng. Group IV taking red ginseng only also showed slope coefficients of 2.0 six months after taking the compound.

Table 7. Change of CD4+ regression coefficients of HIV infected persons by groups and 6 months after taking Korean red ginseng/AZT

Groups	Observed NO.	Before treatment	6 months later
I - A	14	- 19 ± 16.0	48.7 ± 46.6
I - B	42	- 35.7 ± 49.1	7.6 ± 22.0
II	19	- 27.6 ± 36.6	25.3 ± 38.3
III	32	- 12.6 ± 30.1	2.0 ± 48.6
IV	55	- 5.9 ± 16.3	- 5.9 ± 16.3

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

On the other hand, both group I - A and group II showed plus slope coefficients when CD4+ cells were observed 12 months after taking compounds as shown in Table 8.

Table 8. Change of CD4+ regression coefficients of HIV infected persons by groups 12 months after taking Korean red ginseng/AZT

Groups	Observed NO.	Regression Coefficients
I - A	8	11.4 ± 15.6
I - B	25	- 1.6 ± 15.5
II	20	0.3 ± 20.6
III	26	- 3.7 ± 18.6
IV	42	- 5.4 ± 13.0

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

In the group takong AZT there was rapid decrease of CD4+ cells from 6 months after taking AZT. It is interpretated that from this period mutant HIV strains start to replicate in the individuals taking AZT.

There could be 2 hypothesis on the differences in slope coefficients of CD4+ cells by time between 2 groups. One is the Korean red ginseng possibly inhibit appearance of HIV mutant strains directly. The other is that Korean red ginseng possibly delays the destruction speed of HIV infected CD4+ cells in the host.

During the period of 12 to 24 months after taking compounds, the group I - A only showed plus slope coefficients of CD4+ cells, while all the other groups showed minus coe fficients as shown in Table 9.

Table 9. Change of CD4+ regression coefficients of HIV infected persons by groups 24 months after taking Korean red ginseng/AZT

Groups	Observed NO.	Regression Coefficients
I - A	7	2.0 ± 4.6
I - B	23	- 4.5 ± 6.1
II	12	- 2.9 ± 10.3
III	16	- 4.5 ± 11.8
IV	29	- 8.4 ± 11.8

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

With the above results obtained, it is presumed that the effects of Korean red ginseng on the person with HIV become weakened with time after taking compounds. However, it is presumed that anti - HIV activity by AZT is prolonged by the addition of Korean red ginseng. Strangely enough, slope coefficients of CD4+ cells in the group with CD4+ cells of 500 to 200 at entry were - 13.2 in AZT group, while all other group showed plus numbers for first 6 months as shown in Table10.

These results confirm other researches that early treatment of AZT in HIV infected persons with CD4+ of 500 to 200 might not be helpful for patients.

Table 10. Change of CD4+ regression coefficients of HIV infected persons with 500 to 200 CD4+ cells 6 months after taking Korean red ginseng/AZT

Groups	Observed NO.	Regression Coefficients
I - A	4	24.7 ± 14.1
I - B	7	14.1 ± 19.0
II	13	- 13.2 ± 23.9
III	15	4.46 ± 35.8
IV	29	1.54 ± 12.6

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

In the 12 months of observation on the same group, group I - A only showed increase of CD4+ cells as shown in Table 11. However, the number of persons included in this analysis are so small that the importance of these data are doubtful.

Table 11. Change of CD4+ regression coefficients of HIV infected persons with 200 to 500 CD4+ cells 12 months after taking AZT/Korean red ginseng

Groups	Observed NO.	Regression Coefficients
I - A	5	14.12 ± 18.4
I - B	16	- 1.3 ± 12.3
II	14	- 0.31 ± 17.8
III	13	- 1.15 ± 12.0
IV	15	- 0.88 ± 8.1

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

Table 13. Oberved numbers and slope coefficients of p24 antigen in plasma during the research period.

Groups	Observed NO.	Mean ± std Dev.
I - A	4	- 1.01 ± 1.79
I - B	10	- 0.01 ± 0.45
II	2	- 1.67 ± 1.88
III	5	- 0.92 ± 2.29
IV	3	0.34 ± 0.31

I - A : Group taking AZT and Korean red ginseng simultaneously.
I - B : Group taking Korean red ginseng after AZT.
II : Group taking AZT only.
III : Group taking Korean red ginseng only
IV : Control group taking none.

Statistical analysis on CD4+ cell changes by months and groups showed that differences of CD4+ cells between group I - A and group II are significiant within 90% confidence intervals by Wilcoxon scores rank sums test until 6, 12 and 24 months after taking compounds. On the other hand, differences of CD4+ cells among group III and group IV were not found significant by the same test.

Change of β_2- microglobulin.

β_2- microglobulin was tested to monitor every 6 months after taking AZT or Korean red ginseng. Slope coefficients of β_2 - microgolbulins by time and groups are shown in Table 12. Decrease of β_2- microglobulin in group I - A was the biggest compared with those of other groups.

The significance of the differences of β_2- microglobulin among groups is difficult to interprete.

Change of p 24 antigen in sereum/plasma

Concentration of p24 antigen in plasma was tested after taking compound by time. As shown in Table 13, p24 antigen was decreased when AZT or Korean red ginseng was taken. There were no significant differences among groups taking AZT or the ginseng in p24 antigen. However, the numbers observed are so few that exact relationship of slope coefficients and p24 antigen change in plasma is difficult to understand.

Table 12. Observed numbers and slope coefficients of β_2- microglobulin by months after taking AZT/Korean red ginseng.

Groups	6 months		12 months	
	Observed No.	Mean coeff.	Observed No.	Mean coeff.
I - A	9	- 90.8	8	- 85.5
I - B	7	- 6.7	17	- 42.3
II	6	- 22.4	7	- 14.8
III	29	- 16.8	25	- 7.0
IV	36	- 5.8	28	- 1.8

Conclusion

As results of *in virto* anti - HIV inhibition test with fractions of Korean red ginseng and testing on immunological and virological surrogate markers of 193 persons with HIV, the following conclusions were obtained.

- Saponin fraction from Korean red ginseng showed low level of anti - HIV activity *in vitro* MT - 4 cell culture.
- Means of slope coefficients of CD4+ cells in the group taking Korean red ginseng and AZT simultaneously were singificantly greater than of other groups.
- The group taking Korean red ginseng only did not show any significant differences of CD4+ cells from the group takong none.
- The effects of Korean red ginseng and AZT on infected persons seem to decrease with time from around one year after taking compounds.