Stimulatory Effect of Saponin from *Panax ginseng* on Immune Function of Lymphocytes in the Elderly

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ABSTRACT

We used the saponin Rg_1 extracted from *Panax ginseng* to study its effects on lymphocytes of 10 young and 19 elderly persons. The proliferative response of lymphocytes cocultured for 72h with PHA and saponin was measured by using MTT method and the ^{3}H-TdR incorporation procedure. PHA and Rg_1 had stimulative effects on the phenotype of lymphocytes ($p<0.001$). Rg_1 also increased the fluidity of lymphocyte membrane of the aged ($p<0.001$). The CD_{25} and $CD_{45}RA$ positive cells of lymphocytes in the elderly were lower than those of the young people, 8.6% ±2.7% *vs* 10.43% ± 3.5%, 20.95% ±15.5% *vs* 50.86% ±4.3%, respectively. More $CD_{45}RO$ positive cell lymphocyte populations were seen in the aged. The $CE_{45}RO$ positive cells of the young people were 39.63% ± 3.2%. We discussed the cause of declined immune function of lymphocytes of aged person and the mechanism of the effect of *P. ginseng* on lymphocytes.

Key words: Saponin, Lymphocytes, Aged person, Stimulatory effect, *Panax ginseng*

Introduction

It is known that aging is a declining process associated with dysfunction of neuro-endocrino-system network [1]. The thymus is the central organ of immune system, and its atrophy plays a critical role in aging with decreased lymphocyte function. So the immunostimulatory effects of anti-aging tonic drugs have been evaluated as an important indicator. Researchers have successfully studied the effects of Chinese medicinal herbs on animals and human beings. They have determined the effects of mixtures of herbs clinically, but it is difficult to draw a definite conclusion on the mechanism of action of herbs *in vivo*. In this study, Rg_1 extracted from *P. ginseng* was used to study the direct effect of its purified components on lymphocytes of aged people. This study was intended to explore the changes of immune function of lymphocytes in the elderly and the regulatory effect of herbs, and to find a new approach of immunodetecting and immunomodulating of the aging process based on the results of proliferative response of lymphocyte to mitogen, the changes of phenotype, and fluidity of lymphocyte membrane.

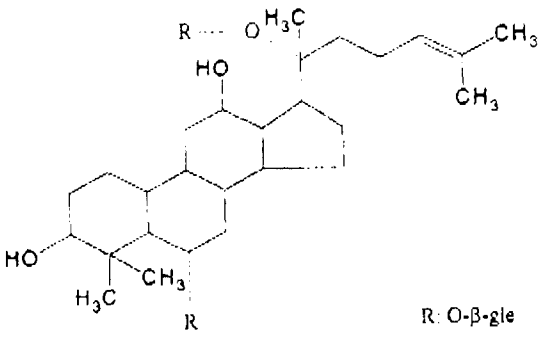
Materials and Methods

Subjects

Nineteen elderly persons (male 8, female 11), aged from 65 to 78 years (mean 68.1), from eastern Beijing were selected, They met the criteria of the SENIEUR protocol through physical examination [2] The young male volunteers (25-30 years) without pathological conditions and immunological abnormalities were taken as controls.

RGI [3]

Rg_1, a saponin extracted from *P. ginseng*, was supplied by the Institute of Inspection for Biological Products, Ministry of Health, P. R. China. Rg_1 was characterized by the following characteristics: white powder, purity 965, melting point 194-196.5℃, specific rotation +32.0, molecular formula $C_{42}H_{72}O_{14}$, infrared spectrum (KBr)cm =3400,1620 and molecular structure



Isolation and culture of lymphocytes [4]

Heparin-anticoagulated vein blood was diluted one time with sterile normal saline, then it was laid on the surface of Ficoll-Hypaque (3:1) in a test tube and centrifuged at 2000 rev./min for 20 min. After that the loafy layer of lymphocytes in the interface was selected and washed with 5% FCS-Hank's(fetal calf serum, Sigma) solution. The residual red blood cells in lymphocyte pellets were lysed by using 0.17M Tris-NH_4 Cl, solution. The isolated lymphocytes were washed twice with 5% FCS-Hank's solution. A cell suspension containing 1×10^6 cells/ml was prepared with RPMI 1640(Gibco) and added to a sterile culture plate 200μg/well. Each sample had triplicate wells. A saponin solution (1 mg/ml) was prepared with RPMI 1640 and sterilized through a 0.22 μg/ml ultrafilter. It was added into each of the three wells at different final concentrations from 0.001 μg/ml to 100 μg/ml. RPMI 1640 without saponin was added in control wells.

Lymphocyte stimulation assay

The isolated lymphocytes were cultured for 72 h at 37℃ in a 5% CO_2 incubator in the presence of PHA-P (phytohemagglutinin, Serva, 5 μg/ml) with RPMI 1640 media containing 25mM Hepes, 5×

10^{-5} M, 2-ME (2-Mercaptoenthanol, Eerch- schuchardt), 10% FCS, 2mM L-glutamine (Sigma) at a 96-well plate in 200μg each well. Rg_1 was presented in triplicate experimental wells at a final concentration of 1 μg/ml. Six hours prior to the end of culture, 18.5KBq of ^{3}H-thymidine uptake was determined in a Beckman scintillation counter, and the results were expressed as counts/min (cpm).

The proliferative response of lymphocytes was also determined by the Mosmann method [5]. Briefly, 200 μg of freshly prepared MTT (3-4,5-diamethyl-2-thiazolyl)- 2,5-diphenyl-2h-tetrozolium, Serva) solution (1mg/ml in PBS 0.01M pH 7.2), filtered through 0.22 μm filter, was added into each well after culture for 68h. The cells were returned for another 4h incubation and centrifuged at 1500 rev./min for 6 min. After the supernatant was removed, a mixture of DMSO (dimethyl sulfoxide) and ethanol (1:1) was added and suspended. The optical density (OD) was determined by using a Dynatech MR5000 ELISA reader at a wavelength of 570 nm and 630 nm within 10min.

Detection of lymphocyte phenotype

50 of lymphocyte suspension (1×10^6 cells/ml)in PBS containing 2% FCS in three Eppendorf tubes was first stained with 50 monoclonal antibodies ACT I (Dako) (CD_{25}, 1:100), 50 4KB (Dako) $CD_{45}RA$, 1;40 or 50 UCHL-1 (Dako) ($CD_{45}RO$, 1;50) and left at 4℃ for 30min. The cells were washed with PBS by centrifugation at 1000 rev./min for 10 min. 50 FLTC-sheep anti-mouse IgG (1:3) was added into each tube at 4℃ for 30 min. After washing again, the pelleted cells were suspended in 50 PBS and mixed with equal volume of 2% paraldehyde solution in PBS. The same procedure was used for staining of lymphocytes before and after culture with PHA alone or PHA/Rg_1 in combination. As a negative control, normal mouse serum was utilized. Fluorescence-positive cells were counted by use of FACS or BH2-RFCA Olympus fluorescence microscopy. The percentage of positive cells were determined by subtracting the percentage of fluorescence positive cells with control serum from that of fluorescence-positive cells with monoclonal antibodies.

Determination of lymphocytes membrane fluidity

A mixture of 1ml of lymphocytes suspension (1×10^6 cells/ml) and 1ml of DPH (1,6,diphenyl, 1,3,5-hexatrienc, Sigma) solution (final concentration 1×10^6 M) was kept at 25℃ for 30 min. Polarizing degree was measured by a polarized fluorescence spectrophotometer (wavelength of excitation light 360 nm, slot of excitation light 40 nm, wavelength of fluorescence 430 nm, slot of fluorescence 50 nm), Then, the lymphocyte membrane fluidity was calculated by the Einstein Equation.

Statistical analysis

The data are expressed as the mean$\pm$s ($\bar{x}\pm$s), The Dunnett *t*-test was used for statistical evaluation of the data. $P<0.05$ was considered significant.

Results

Direct bi-directional regulatory effect of saponin on lymphocytes

Table 1 shows the result of saponin using the MTT method. Saponin has a direct stimulatory effect on the lymphocytes of aged people. In the presence of exogenous PHA, saponin can signifi-

Table 1. Effect of Rg_1 on the PHA responsive proliferation lymphocytes in the elderly

MTT method(Wave length 570 nm/630nm)			
cases	PHA[a]	PHA(5μgml)b2 + Rg_1(1μgml)	P value
1	0.1530	0.1947	
2	0.1310	0.1677	
3	0.1425	0.1720	
4	0.1253	0.1393	
5	0.1300	0.1503	p<0.05
6	0.1517	0.1357	
7	0.1300	0.1200	
8	0.1177	0.1590	
9	0.1597	0.1950	
x	0.1379	0.1592	
s	0.0143	0.0258	

Table 2. Dose-dependent bi-directional regulation of Rg_1 on lymphocytes in the elderly

Rg1(μg/ml)	PHA(μg/ml)	^{3}H-TdR uptake $10^3 \times$ counts/min($\bar{x} \pm s$)	P value
0	5	28.4 ± 3.6	
0.01	5	32.6 ± 2.4	
1.0	5	36.0 ± 0.6	P<0.001
	0	1.7 ± 0.4	(a:b)
10.0	5	35.1 ± 1.0	
100.0	5	31.8 ± 5.1	

cantly promote and activate the mitosis of lymphocytes. Three characteristics could be seen in this stimulation:

Dose-dependent bi-directional regulation

Saponin enhanced the proliferation of lymphocytes at optimal concentration (Table 2). Otherwise, the inhibitory effect occurred at a higher or lower concentration of saponin beyound the optimal range. the optimal concentration of Rg_1 was 1μg/ml(0.1-10μg/ml) (Fig 1.) and that of sum total saponin of *P. ginseng* was 10μg/ml. We concluded that action of Rg_1 is stronger that that of sum total saponin

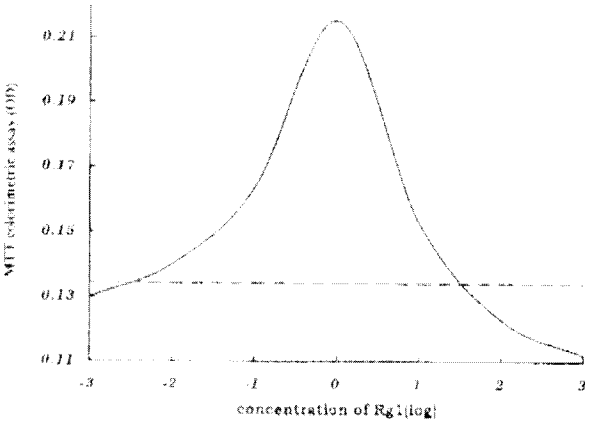
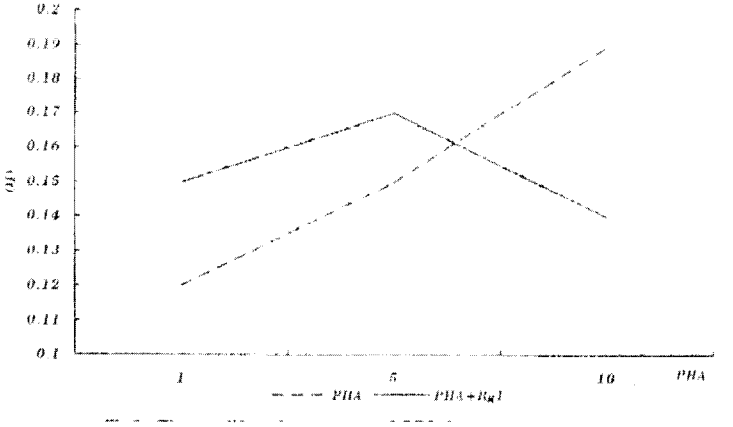


Fig1. Dose-dependent synergistic effects of Rg1 with PHA on proliferation of lymphocytes.



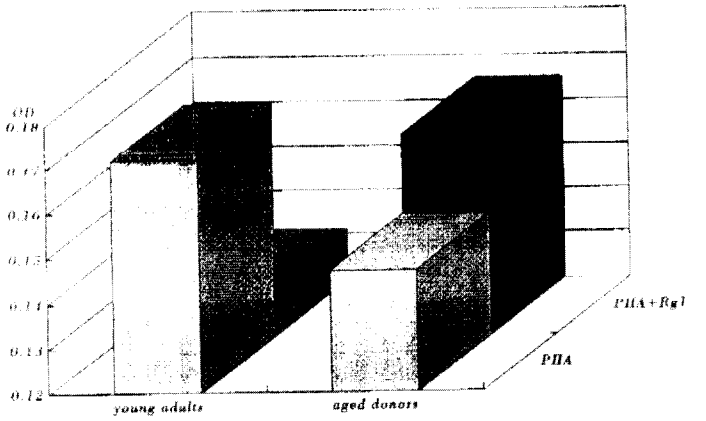


Fig3. Age-related changes in response of PBL to PHA/PHA+Rg1.

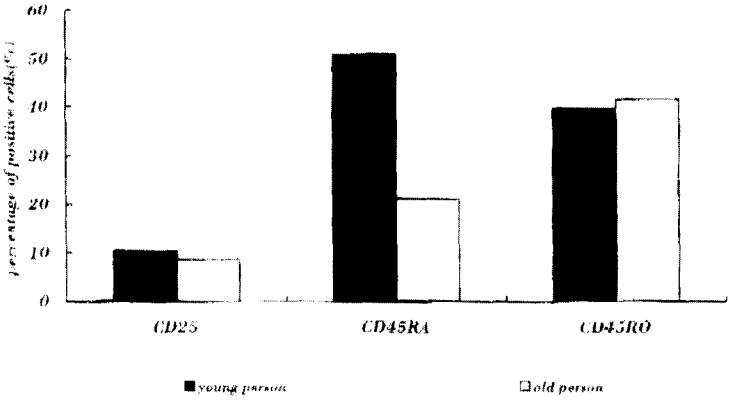


Fig4. Percentage of CD25, CD45RA and CD45RO positive cells in isolated lymphocytes of young and old person.

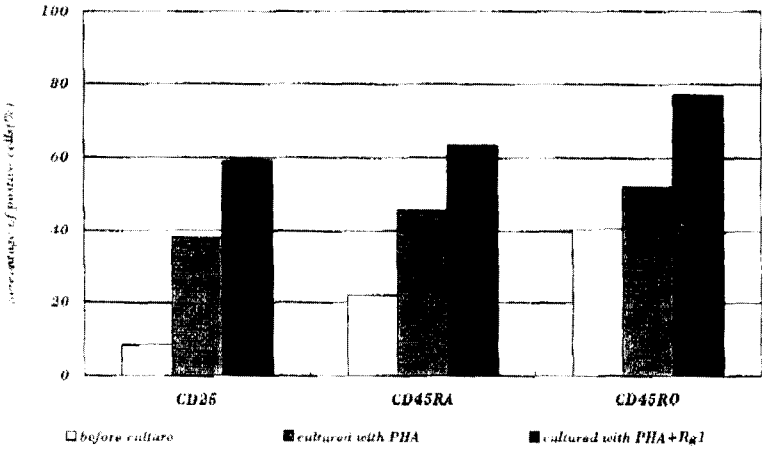


Fig5. The phenotypic expression of PBL from aged donors.

Influence of saponin on phenotype of lymphocytes [6].

Phenotype, CD_{25}, $CD_{45}RA$ and $CD_{45}RO$ of isolated lymphocytes before and after culture with PHA and PHA/Rg_1 for 72 h were examined with monoclonal antibodies, ACT-1, 4KB and UCHL-1, respectively (Fig4. and 5.). The CD positive ($8.6\% \pm 2.7\%$) and $CD_{45}RA$ positive cells ($20.94\% \pm 3.5\%$ and $50.86 \pm 4.2\%$, respectively) of young persons. The $CD_{45}RO$ positive cells were $41.57\% \pm 13.9\%$ in the aged and $39.63\% \pm 3.2\%$ in young persons. The sum of $CD_{45}RA$ and $CD_{45}RO$ positive cells of aged persons were lower than those of the young. Also, PHA and saponin had marked stimulatory effects on lymphocyte phenotype ($P<1.001$).

Table 3. Effect of Rg_1 on the PHA responsive proliferation lymphocytes in the elderly

Subject	Cases	After culture for 72h			P value
		Before culture	PHA	PHA + Rg1	
Young	4	3.95 ± 0.97[a]	4.45 ± 1.29	9.99 ± 1.71[c]	c:a, P<0.001
Old	10	3.07 ± 1.75[b]	3.85 ± 1.75	4.55 ± 1.57[e]	f:d, P<0.050

Effect of saponin on membrane fluidity of lymphocytes [7]

The membrane fluidity of lymphocytes from old people before culture was lower than that of young people, but there was no statistical difference. However, Rg_1 had a definite stimulatory effect on the membrane fluidity of lymphocytes of both aged and young people ($P<0.05$).

Discussion

The fact that the responsiveness of lymphocytes in the elderly declines with aging is well recognized, but the cause of aging is still obscure. Some researchers believe that the decrease response of lymphocytes results from the impaired matabolism associated with many factors. In our study, the membrane fluidity, surface antigen expression, proliferative response of lymphocytes and the percentage of IL-2 receptor positive cells were lower in the elderly than in young persons. The saponin we used can stimulate and enhance the function of lymphocytes, and furthermore, can restore the function to normal.

The proliferative response of activated lymphocytes stimulated with PHA is usually employed for evaluation of cell-mediated immunity. The impaired response of PHA-induced lymphocyte proliferation would be indicator of aging, its degree correlates with age. Lucivero *et al.* [8] studied the kinetic changes of PHA-induced lymphocytes proliferation with ^{3}H-TdR uptake and demonstrated that the intake of ^{3}H-TdR by lymphocytes of aged people after 3-day culture with PHA was significantly lower than that from young person ($P<0.02$). We have achieved similar results. They found that the decreased proliferative response of lymphocytes of aged people is due to obstruction of active signal transduction from cell surface receptor to the inside of the cell, in contrast to reduced number of cells in circulation, different nutritional request of variation of cell viability. The cross-linking and capping of cell surface receptor play a crucial role in the process of activation and proliferation involved by cytoskeleton, i.e. the decline of immune function in the elderly is mainly caused by the dynamic movement of cell membrane and cytoskeleton. Most PHA-stimulated lymphocytes of aged people stayed on G2 and M phase of cell cycle could not become G1, and completes S phase. These investigators noted that the native capping of antigen specific receptor of lymphocytes from cord blood increased and the membrane fluidity of neonatal lymphocytes raised. It is implicated that the cell membrane fluidity can affect signal transduction and cell activation.

Tollefsbol *et al.* [9] confirmed that protein synthesis of lymphocytes from aged people after stimulation for 24-72 h with PHA is going down. In this period, the intake of radiolabeled leucine of lymphocytes of aged people is just half of that of young people. They determined that the low ability of protein synthesis is related to dysfunction of induction of the glycolytic enzyme needed in mitosis of lymphocyte in the elderly, the components for growth and proliferation such as adenopurine and pyridine nucleotides of glycolytic products involved in lymphocyte transformation are absent. They consider this event a molecular basis for explanation of the decreased proliferation of lymphocytes in the elderly.

This conclusion provides some insights to explore the immunostimulatory mechanism of saponin and other herbs. Saponin is a polysaccharide with specific characteristics. Ginseng contains more than 10 saponins. Some researchers found that ginseng can promote synthesis of protein, RNA and DNA in various organs or tissues, for example, liver, kidney, bone marrow and plasma of rat. The saponin components of ginseng like Rc, Rc_2, Rd and Rg_1 can increase the incorporative rate of ^{3}H-leucine into protein of mouse serum, especially Rg_1 that can stimulate the mitosis of lymphocytes. Enhancing the membrane fluidity of lymphocytes by saponin, in our study we supported the hypothesis that the interaction of saponin with lymphocytes might be accomplished through a series of complicated processes, including cell membrane fluidity, structure of phenotypic antigen and receptor, and protein synthesis.

Some researchers [10] found that the proliferative response with PHA and the production of bioactive IL-2 of lymphocytes from young people were two times higher than those of aged people. Because the degree of proliferation of activated lymphocytes is associated with the amount of IL-2, less production of IL-2 by lymphocytes of aged people limits their proliferation. However, exogenous IL-2 by even biologically excessive amounts of IL-2 can not totally restore the reduced proliferative activity of lymphocytes of aged people. This result suggests that the decreased produdtion of IL-2 may be accompanied by deficiency of IL-2 receptor. Chopra *et al.* [11] analyzed the lymphocytes of aged people by cytometry using fluorescence-labeled anti-CD_{25} antibody, and found that the percentage of IL-2R positive cells in aged people was significantly lower than that in young people. Similarly, the shortage of high affinity IL-2R in old mice caused a dramatic decline of IL-2-induced cell proliferation. Our findings supported this conclusion. Recent research indicated that both IL-2R expression and IL-2 production are not only important for entry of cell into S phase, but also for going through G phase prior to triggering DNA synthesis of activated lymphocytes.

The phenotype of non-responsive lymphocytes of aged people is not known. Some researchers reported that the ratio of $CD_4^+CD_{45}RA^+$cell (native cell over) $CD_4^+CDw_{29}^+$cell (memory cell) of aged people and animals was sharply decreased. Animal experiments revealed that old mice have much more memory cell, and that the ^{3}H-TdR uptake of the cells is low. The fact that the PHA responsive ability of $CD_4^+CD_{45}RA^+$cells is higher than $CD_4^+CDw_{29}^+$ cells lead us to find the real cause of

impaired immune response in the elderly. The double color flow cytometry examination showed that the antigen specific native $CD_4^+CD_{45}RA^+$ cells of over 60 years old people apparently decreased to 24%. In contrase, the $CD_4^+CD_{45}RO^+$ memory cells increased to 33%. The extra memory cells should consist of 'true' (matured) and 'false' (immatured)cells. In our study, we found that the $CD_{45}RA^+$ cells and $CD_{45}RO^+$ cells simultancously elevated after co-stimulation with PHA and Rg_1. Since we did not detect the separated cell sub-population, the sum of $CD_{45}RA$+ plus $CD_{45}RO^+$ cells surpassed 100% owing to the co-expression of two phenotypes on CD_8^+ cell surface.

Scholars described the cause of declined secretion of IL-2. The IL-2R chain mRNA in the lymphocytes of aged people decreased to 1/3 of the young people. It means that research work on cellular and molecular level should be done to clarigy the mechanism of saponin. Therefore, whether saponin can act on IL-2 or IL-2R gene expression of lymphocytes in the elderly would be the research subject in the future.

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