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Growth Promoting Activity of a Macromolecular Fraction from Fresh Ginseng

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Abstract□ 1) A macromolecular fraction from fresh ginseng root containing mainly polysaccharide showed mild mitogenic activity in human cord blood lymphocytes. 2) When lymphocytes were transformed by Con A or PHA, this fraction could greatly enhance the activity of these lectin mitogens, thus showing a potentiation effect. 3) Although this macromolecular fraction contains saponin and is susceptible to trypsin digestion, it is probably a peptido-glycan in nature on account of its important carbohydrate content and thermal stability. 4) This fraction could not support cancer cell (EAT, K562) growth; its inhibitory effect on these cells remains to be explored.

Keywords□*Panax ginseng* C.A. Meyer, polysaccharide, mitogenic activity.

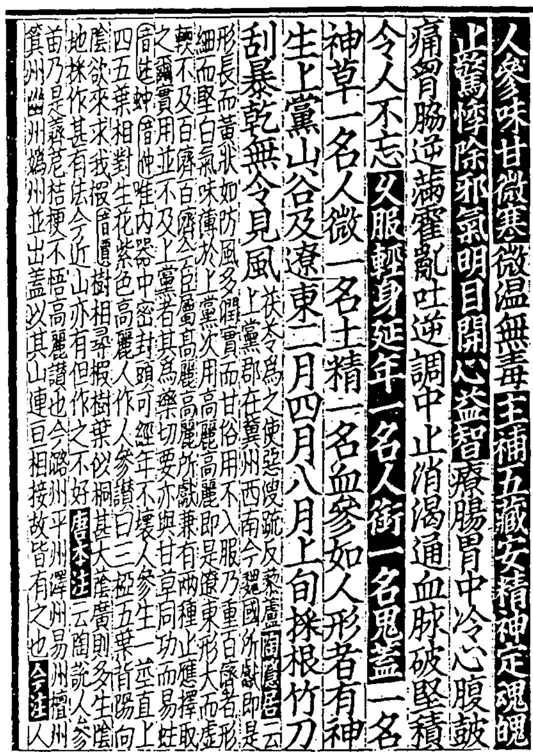
Introduction

Ginseng (Fig. 1) occupies a special position among Chinese medicinal plants because it has a wide-spectrum tonic action. After ginseng saponins were discovered due to the pioneer works of Shibata and continued with much success by Tanaka[1], much of the pharmacological properties of ginseng were ascribed to its ginsenoside components. Before there was any laboratory study like what we are doing today, the Chinese people has long since summarised their observations on the biological effects of ginseng in *Shen Nung Pents'ao* dated around 200 A.D. (Fig. 2) Here it described a sedative and nerve calming effect, a tonic effect for visceral and neural functions and a beneficial property to ward off diseases. These effects maybe in one way or another related to the pharmacological properties of ginsenosides. But it also mentioned that the chronic user of ginseng will develop a light body and enjoy longevity. Previously this is just put off as an ideal situation in taoist practice as *Shen Nung Pents'ao* was probably the collecitve work of *Han* taoists and they used more than one Chinese medicinal plant for similar benefits. With new knowledges in nutrition and immunology, we now know that if we want to live long and healthy, we must control our body weight and we must have a strong immuno-defense capacity so that we are less prone to develop matabolic diseases (like obesity induced complications) and less prone to suffer from virulent infections due to a weakening immuno-defense system, especially in senility.

Therefore, it is fair to say that ginseng may produce some long term beneficial effects that we have so far been unable to reveal by short-term laboratory experiments at the cellular and sub-cellular level. Ginseng may produce a state of well-being at the organismic level that cannot be explained by chronic loading with ginseng saponins or quantitatively measured by physiological parameters.

While ginsenosides remain the focus of ginseng studies, recently many compounds were discovered in ginseng with interesting biological activities. There are phenolic compounds with anti-oxidant effect[2], anti-lipolytic peptide[3], alkaloids[4], fatty acids and esters[5], polyacetylenes[6] and polysaccharide with anti-complementary activities[7]. A polysaccharide of 1.5×10^6 m.w. that could potentiate reticulo-endothelial system activity was found in

Fig. 1. An inconograph of ginseng from *Ta Kuan Pents'ao* (1108 A.D.) This refers to the genuine ginseng grown in the prefecture of *Lu* (nowadays Shansi Province) which had been since then exhausted and was replaced by *Tang Seng* (*Codonopsis pilosula*). From the same page three other plants called "ginseng" were also presented.



人參味甘微寒微溫無毒主補五藏安精神定魂魄止驚悸除邪氣明目開心益智療腸胃中冷心腹鼓痛胸脇逆滿霍亂吐逆調中止消渴通血脈破堅積令人不忘久服輕身延年一名人銜一名鬼蓋一名神草一名人微一名土精一名血參如人形者有神生上黨山谷及遼東二月四月八月上旬採根竹刀刮暴乾無令見風茯苓為之使惡溲疏反藜蘆陶隱居云上黨郡在冀州西南今魏國所獻即是形長而黃狀如防風多潤實而甘俗用不入服乃重百濟者形細而堅白氣味薄於上黨次用高麗高麗即是遼東形大而虛軟不及百濟百濟今臣屬高麗高麗所獻兼有兩種止應擇取之爾實用並不及上黨者其為藥切要亦與甘草同功而易蛀（音注）蚛（音仲）唯內器中密封頭可經年不壞人參生一莖直上四五葉相對生花紫色高麗人作人參讚曰三椏五葉背陽向陰欲來求我椵（音賈）樹相尋椵樹葉似桐甚大陰廣則多生陰地採作甚有法今近山亦有但作之不好唐本注云陶說人參苗乃是薺苨桔梗不悟高麗讚也今潞州平州澤州易州檀州箕州幽州媯州並出蓋以其山連亘相接故皆有之也今注人

Fig. 2. Description of ginseng in *Ta Kuan Pents'ao*. The large white characters on a black background was a transcription from *Shen Nung Pents'ao* dated late *Han* dynasty (200 A.D.)

Sanchi-ginseng[8]. There are also hypoglycemic glycans[9] with large molecular weight (1.8×10^6) and little peptide content (0.7%)[10] and a group of smaller glycans with more peptide content (0.7%)[10] and a group of smaller sglycans with more peptide content (3.4-10.3%)[11-13]. This rapidly expanding horizon of ginseng phytochemistry and pharmacology encourages one to look for bioactive polar macromolecules in ginseng.

It may be appropriate to make it clear that we always treat ginseng as a medicinal plant and not a commodity. We believe that if we want to delive ginseng as a valuable commodity or an effective therapeutic agent, we must fully understand its chemistry and pharmacology in its natural state. We cannot go on promoting the use of ginseng by virtue of its folklore. Thus we prefer to work on fresh ginseng, whether frozen immediately after harvest or oven-dried under laboratory conditions. We have studied the saponin content of fresh ginseng from China[14] and Nepal[15], and now continue with this approach to study the macromolecular fraction of fresh ginseng from Korea.

Materials and Methods

Fractionation and chemical determination

The fractionation scheme was designed to obtain ginseng polysaccharide in its native state (Fig. 3).. Thus fresh ginseng (4 year roots from Kum San area) was blended twice with an equal volume of distilled water (1:1 w/v) and passed through gauze. The aqueous extract was allowed to settle overnight

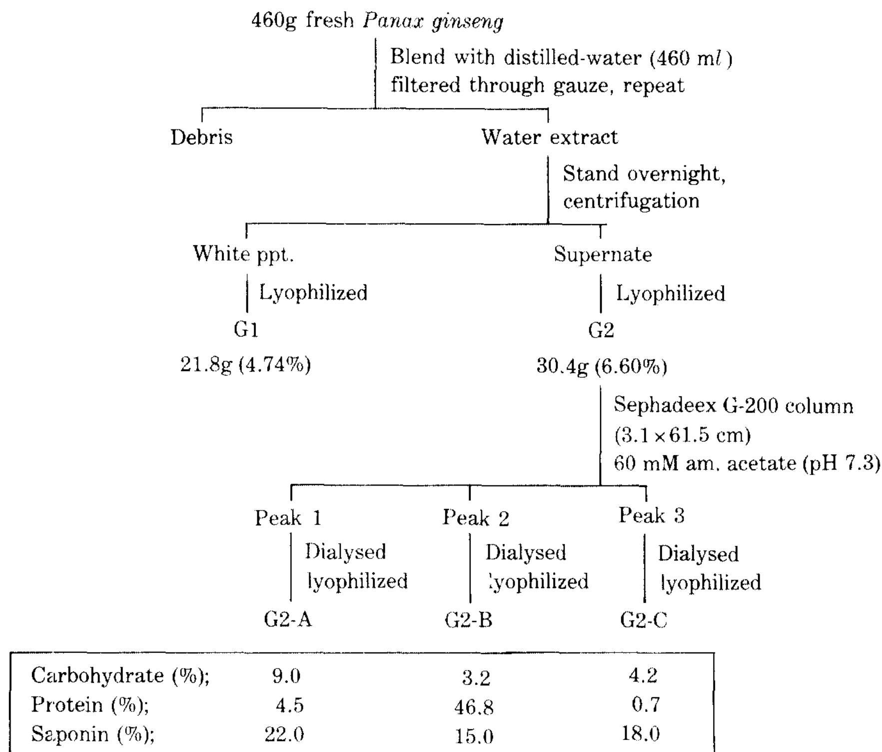


Fig. 3. Fractionation scheme of ginseng polysaccharide for bioassay.

to remove the fine particles and a large part of the starch precipitate with further clearance by 10,000g, 30 min. The supernate was lyophilised and small aliquots (1-2g) were reconstituted in dilute acetic buffer (pH 7.3) to be processed by Sephadex G-200 column into 3 main peaks (Fig. 4). Protein, carbohydrate and saponin contents were determined by Bio-Red protein assay, anthrone-sulphuric acid method and vanilline-sulphuric acid method, respetively.

Cell culture

Human peripheral blood lymphocytes (PBL) was obtained from heparinized umbilical cord blood[16]. 1×10^5 PBL in 0.1 ml culture medium [RPMI 1640 (GIBCO) medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (GIBCO) and 1% antibiotics (GIBCO)] were added to each well of a flat-bottom 96-wells culture plate (CORNING). All samples were tested in triplicate wells. Murine splenocytes were prepared from the spleen of BALB/c mouse bred in the University Animal House. Spleens from groups of 3 to 4 mice were removed aseptically, minced with a pair of scissors and pressed through a sterile 60 mesh stainless steel screen with the plunger of a plastic 5 ml syringe. Large cell clumps were removed by centrifugating at 300 g for 10 sec. The cells were then pelleted and resuspended in Tris-NH_4Cl buffer at room temperature for 5 min to lyse red blood cells in order to facilitate cell counting. The cell suspension was then centrifuged at 300 g and washed three times with RPMI medium. Variable spleen cells (5×10^5/well) were cultured with G2 fractions

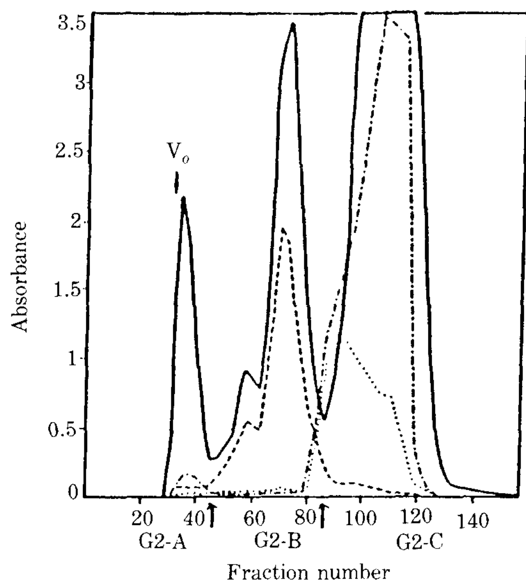


Fig. 4. Elution profile of G2 on Sephadex G-200 fine column (column size, 3.1×61.5 cm; flow rate, 4 ml/20 min. eluent, 60 mM NH_4Ac; sample, 2g/25 ml).
—, A_{280}nm; - - -, BioRad protein, A_{595}nm; ······, Saponin, A_{545}nm; -·-·-, Carbohydrate, A_{480}nm.

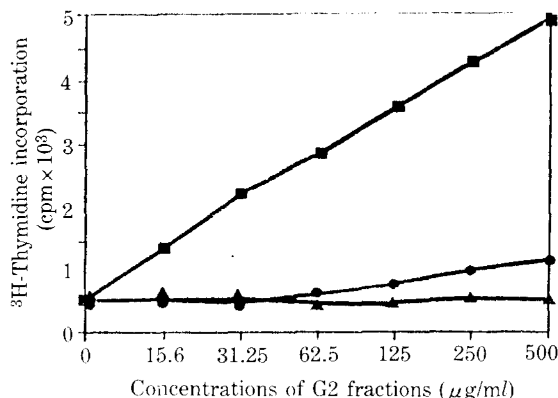


Fig. 5. Mitogenic effect of G2 fractions from Sephadex G-200 column on human PBL (■, G2-A; ●, G2-B; ▲, G2-C).

in 0.2 ml RPMI culture medium in flatbottom 96-well plates. The different fractions of G2 were dissolved in phosphate buffered saline (PBS) and filtered through Millipore filter. Con A (50 μl) or other mitogens dissolved in culture medium was added. The cultures were then labelled for 6 hrs with [methyl-^{3}H] thymidine (Amersham 1 mCi/ml) [2.0 Ci/mmol] at 0.5 μCi per well. The cells were harvested with a cell harvester (Dynatech, Automash 2000). The radioactivity was counted in a liquid scintillation counter (Beckman) and was presented as the mean of 3 close triplicates. The EAT (Ehrlich Ascites tomour), K562 (Erythroleukemia cell line) cells (1×10^4/well) in 100 μl RPMI 1640 culture medium were seeded into 96-wells plate. G2-A (100 ul) dissolved in PBS was added to the culture and incubated in CO_2 incubator for 1 day and then pulsed, harvested and counted as mentioned above.

Results and Discussion

The first peak (G2-A) was eluted near the void volume and it contained mainly polysaccharide with molecular weight at the exclusion limit (200K) of G-200. The second peak (G2-B) was eluted at mid-retention volume; it was mainly protein. The third peak (G2-C) was eluted at total retention volume; it was mainly saponin and oligosaccharides (Fig. 5). All three peaks were pooled, dialysed and lyophilised. Aliquots from each fraction were reconstituted in culture medium and passed through Millipore filter for bioassay. Since the lyophylised powder could not be completely redissolved in the culture medium at higher concentrations than 2 mg/ml at room temperature, the Millipore filtrates were again checked for their chemical contents that were actually used in cell culture. Results showed that they were very little different from the prefiltration samples. While it could be confirmed that G2-A was polysaccharide and G2-B was protein, saponin was found in all frations at more or less the same concentration. Since the pooled fractions had been dialysed, it is reasonable to assume that these are bound saponins attached to large polysaccharide and protein molecules. Saponins are known to interact with proteins and enzymes resulting in a change of conformation and activity[17]. Saponins may also exist as micelles with an apparent mol. wt. of 100K (personal communication, Prof. Tanaka).

When human PBL were treated with these fractions on the weight scale, G2-A showed mitogenic

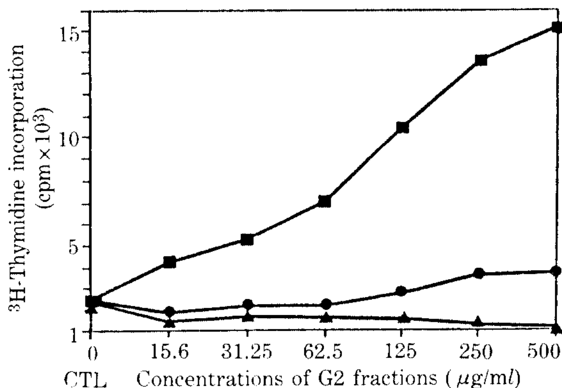


Fig. 6. Co-mitogenic effect of G2 fractions from Sephadex G-200 column with Con A 0.5 μg/ml on human PBL (■, G2-A; ●, G2-B; ▲, G2-C).

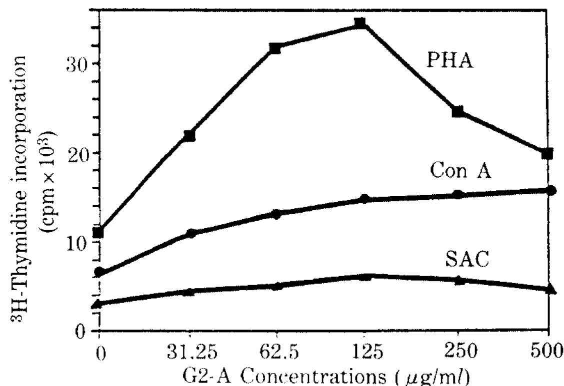


Fig. 8. Co-mitogenic effects of fraction G2-A with different mitogens on human PBL (●, Con A 0.5 μg/ml; ■, PHA 0.125 μg/ml; ▲, SAC 50 μg/ml).

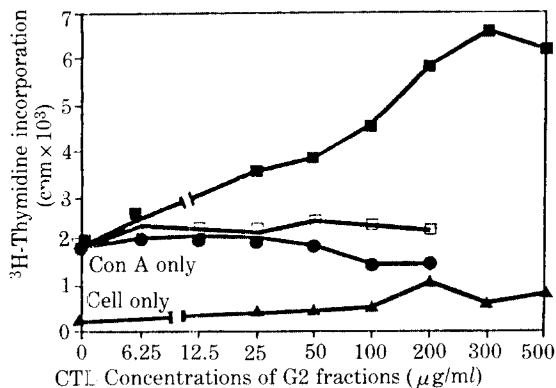


Fig. 7. Mitogenic effect of fraction G2-A and co-mitogenic effect of G2 fractions separated by Sephadex G-200 column with Con A 0.5 μg/ml on human PBL (▲, G2-A only; ■, G2-A+Con A; □, G2-B+Con A; ●, G2-C+Con A).

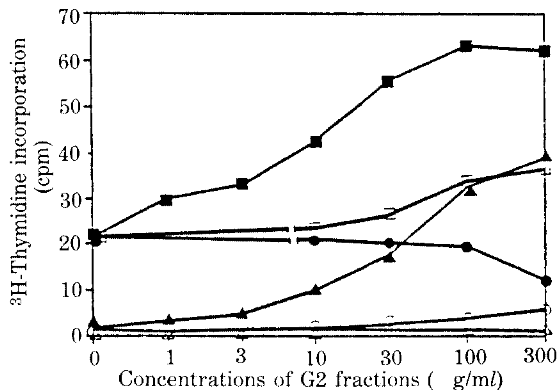


Fig. 9. Mitogenic and co-mitogenic effects of G2 fractions on murine splenocytes (▲, G2-A; ○, G2-B; △, G2-C; ■, G2-A+Con A 0.25 μg/ml; □, G2-B+Con A 0.25; ●, G2-C+Con A 0.25 μg/ml).

activity by stimulating a 10-fold increase in thymidine incorporation. G2-B was slightly active at very high concentrations and G2-C was not active at all (Fig. 5). Since all three fractions contained more or less the same amount of saponin, it is clear that the mitogenic activity may be ascribed to the polysaccharide component in G2-A, unless a unique saponin bound to polysaccharide or somehow non-dialyzable and different from those saponins in G2-B and G2-C might exert this activity pattern.

The same activity pattern appeared in Con A transformed lymphocytes (Fig. 6). Here G2-A could further enhance Con A stimulation by 8-fold and G2-B was slightly active at high concentration. G2-C was not active at all, it had weakened part of the Con A stimulation. Here the same reasoning goes to the role played by saponins and later on we showed that saponins were not active in this bioassay system.

In order to show the potentiation effect of G2-A on Con A stimulation, mitogenic and co-mitogenic activities were demonstrated in the same experiment (Fig. 7). Concurrently active *per se*, G2-A in the presence of Con A, enhanced thymidine incorporation beyond their additive value especially at higher concentration. This potentiation effect was also observed with PHA but not with SAC (*Staphylococcus aureus* Cowan) (Fig. 8).

Murine splenocytes were tested with G2 frac-

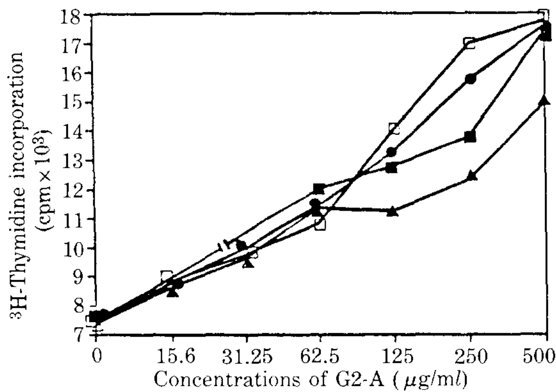


Fig. 10. Biological activity of fraction G2-A after solvent perturbation (■, G2-A untreated; ●, G2-A with BuOH; □, G2-A with acetone; ▲, G2-A with dioxane)

tions and their activity patterns were the same as in human lymphocytes (Fig. 9). Here G2-A was strongly mitogenic and co-mitogenic but with the latter activity, an additive effect was observed instead of a potentiating effect as in human lymphocytes. Perhaps this could be explained by the fact that splenocytes contain a larger population of B-cells comparing to peripheral lymphocytes and G2-A potentiation was observed with Con A and PHA but not SAC as shown above.

Con A and PHA are known to be T-cell mitogens while SAC is B-cell mitogen. It is fair to conclude from these data that G2-A containing mainly macromolecular polysaccharide was active as a mitogen *per se* in T and B cells and it could greatly potentiate the activity of lectin mitogens in human lymphocyte T-cells.

Since we are concerned with the presence of saponin in G2-A and G2-B, we try to find out whether these bound or micellar saponins contribute to lymphocyte activation. Thus water miscible organic solvent (dioxane, acetone or alcohol) was added to aliquots of reconstituted fractions at 15% in order to promote the dissociation of bound saponins without denaturing the macromolecular carrier. After 4 hours of incubation at room temperature, the organic solvents were removed by rotary evaporator and the treated fractions were first lyophylised and then dissolved in culture medium for bioassay. Fig. 10 showed that organic

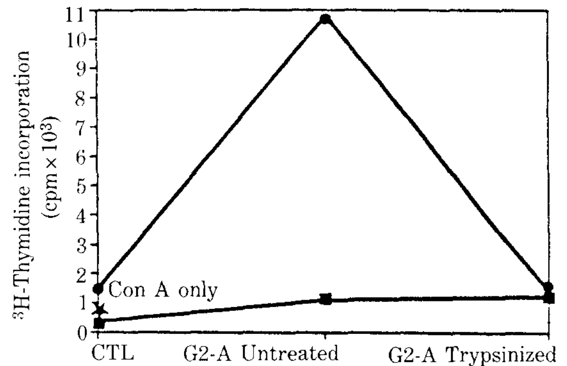


Fig. 11. Biological activity of fraction G2-A after trypsinization (0.33% trypsin) (■, G2-A only; ●, G2-A + Con A 0.5 μg/ml; ★, Con A 0.5 μg/ml with inactivated trypsin solution).
Trypsin was inactived by heating at 80°C for 10' followed by addition of trypsin inhibitor (1:3.5).

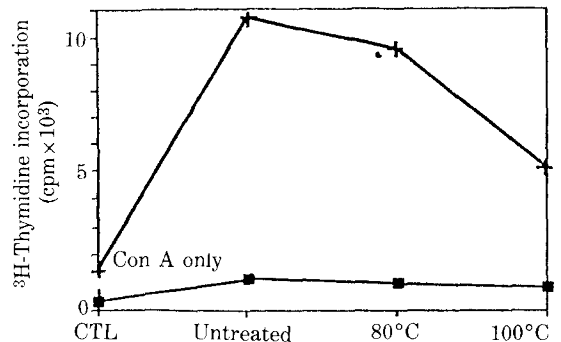


Fig. 12. Potentiation effect of fraction G2-A after thermal inactivation (+, G2-A 200 μg/ml + Con A 0.5 μg/ml; ■, G2-A 200 μg/ml only).
Samples heated at 100°C had been previously treated at 80°C, 10' in a water bath with sample stirring.

solvent perturbation had little influence on the co-mitogenic activity of G2-A.

Since saponins remained in the sample after solvent perturbation, it would be interesting to see if pure saponins can potentiate Con A stimulation. Lymphocytes were concurrently activatd by Con A and saponins by adding ginsenosides Re, Rb_1, Rb_2 isolated in this laboratory to the cell culture to an equivalent amount (100 μg/ml) of its content in the fractions tested (15-22% of 500 μg/ml). Results showed that neither panaxatriol (Re) nor panaxadiols (Rb_1, Rb_2) could potentiate Con A stimulation

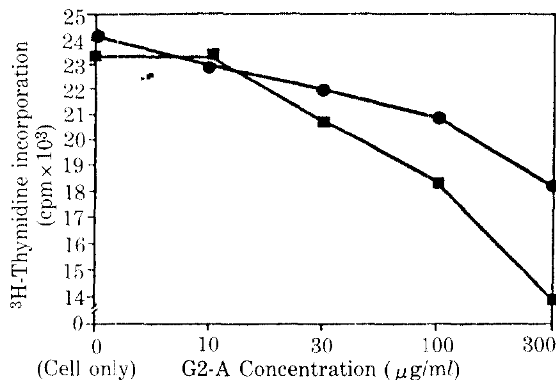


Fig. 13. Effect of fraction G2-A on thymidine incorporation in tumor cells cultures (●, EAT cell; ■, K562 cell).

Table 1. Absence of co-mitogenic effect of ginsenosides on human PBL activated by Con A.

	0 (CTL)	3	10	30	100 (g/ml)
Re	3870	3701	3533	3773	3953
Rb_1		3888	3392	2989	3575
Rb_2		3784	2265	2467	2306

(Cells were incubated with Con A 0.5 g/ml and ginsenosides for 3 days.)
Control = Con A only

of human lymphocytes (Table 1).

Preliminary attempts to characterise the polysaccharide nature of G2-A showed that its co-mitogenic activity could be totally abolished by trypsinisation prior to its addition to cell culture (Fig. 11). This indicates a fairly important peptide moiety (3.2% peptide in undialyzed sample) in the polysaccharide component and it is indispensable for its biological activity. However, a good thermal stability of G2-A (Fig. 12) showed that it is basically a polysaccharide as a protein of this molecular weight range (200K) would be easily denatured by heat treatment. Further attempts to characterise the chemical nature of G2-A must wait for purer and more homogeneous preparations.

Immuno-modulating effect of ginseng polysaccharide is of course an interesting attribute to the beneficial properties of ginseng. But a mitogenic effect would necessarily solicit the question of its carcinogenicity or a strong co-mitogenic effect, its possiblity of promoting existing cancer growth. So far we have observed that G2-A could not support the growth of Ehrlich ascite tumor cells and an erythroluekemia cell line (Fig. 13). The inhibitory effect at higher concentrations remains to be explored, but at this stage, we are satisfied that ginseng polysaccharide will not make cancers worse. A similar situation was observed with a mitogenic iso-agglutinin from alga, it has anti-neoplastic effect[18]. We are also not yet clear at this stage if this immuno-modulating polysaccharide is unique to ginseng. Other polysaccharide immuno-modulators from plants and fungi have been reported[19]. We cannot totally exclude the effect of contaminating endotoxin as the ginseng used was fresh with a trace of soil. However, lipopolysaccharide mitogens are B-cell active and it would be unlikely to show a potentiating effect in human lymphocytes.

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