발표초록 모음

A Resume of Biotechnology in China

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China has made remarkable contribution to mankind on the utilization of biological resources for the development of agriculture, medicine, fermented food and beverages, After the establishment of the people's Republic of China, modern fermentation technology was first adapted in the development of antibiotic industory. Production of MSG, enzymes, citric acid, vitamin B_2, vitamin C, steroid hormones, organic solvents and food yeast was subsequently started. In addition, the traditional brewing has also undergone modernization by using pure cultures, strain breeding, strict sterilization and sanitation, so as to increase both the yield and quality. At present, the total output value of Chiness bioindustry is about one persent of the value of gross national product.

In this report, the present status of Chinese biotechnology will be briefly presented to include:

1. Government policies on the development of biotechnology.
2. The Seventh-Five Year Plans, Eighth-Five Year Plans and High Technology Program.
3. Present status of fermentation industry.

Present Status of Fermentation Industries in Korea

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Fermentation industries in Korea have been continuously developed during the last quater of a century. At the early stage of the industrial development, several fermentation industries were only capable of producing fermented foods and alcoholic beverage. The related technology and experience on these bases have been extended to develop modern fermentation technologies for the pruduction of various amino acids, antibiotics and enzymes which are significant for industrial applications.

The major fermentation products in Korea are alcoholic beverages, amino, acids, antibiotics, enzymes, and fermented food products using raw materials such as milk, soybean and various vegetable souces. Among these, amino acids such as monosodium glutamate, lysine, phenylalanine can be recognized as major items produced by applying the modern fermentation technology. Also, Rifampicin is the most important therapeutic drug produced in Korea, and their production technologies have been developed by domestic pharmaceutical industries and research institues since 1970.

The whole production scale of fermentation procucts in Koreas was about 41,000 billion won (90) which was equivalent to 2.% of the total GNP of the same year.

In this report, the recent status of fermentation industries and R & D activities in Korea will be reviewed.

Gentic Engineering Production and Protein Engineering of Pharmaceutical Proteins

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Pharmaceutical proteins produced and studied by genetic engineering of protein engineering method in our lab were interleukins, interferons, tumor necrosis factor, GM-colony stimulating factor and so on.

I) Genetic Engineering Production of Natural Interleukin-2 and its clinical trials.

In the 12 species of interleukins, the Interleukin-2 (IL-2) was the one stuied more intensively because of its potentiality in clinical use. High level expression of recombinant natural IL-2 (r-IL-2) was caried out in this lab. The level of rIL-2 expressed in *E. coli* amounted to 35-40% of the total bacterial proteins. In large scale fermentation 300-500 mg per liter could be reached with IL-2 still amounted to 40-50% of the total bacterial proteins. As to purification, a two main step procedure of the isolation of inclusion bodies and the chromatography on Sephacryl S-200 could purify IL-2 to a purity more than 96%. This technique in large scale production of IL-2 is very simple and fast. In collaboration with Military Academy of Medical Sciences in pilot production, we can produce more than 1.0g of rIL-2 from 10L fermentation medium in about 10 days by about 10 scientific workers. In accordance with the retail price IL-2, more than several million dallors could be easily worked out. Because of the good quality, our rIL-2 has been approved by the Chinese Ministry of Public Health for clinicla trial. The more important thing is that many good results for the clinic use of rIL-2 have been obtained. In the treatment of chronic active hepatitis B9CAH disease, the therapeutic effect reached 53-70%. Up to date, the more effective drug for HBV disease therapy is α-interferon (IFM-α), but the anti CAH effect of rIL-2 is better than IFN-α, especially for the long term effect, and the cost for rIL-2 in the CAH therapy is 10 times lower than that of IFN-α, IL-2 is also very useful for the treatment of malignant tumor. The effect for the elimination of malignant pleural effusion from lung cancer reached 90%, for the elimination ascite caused by advanced ovarian cancer reached about 80%. IL-2 is also useful to the treatment of lung cancer, melanoma, renal carcinoma, blood cancer, hepatoma, nasopharynx cancer with different effects. In 1985, Rosenberg had treated 25 patients with IL-2/LAK and 11 cases got CR, the total response was 44% which attracted a worldwide interest in the IL-2/LAK therapy. But the therapeutic effect of IL-2/LAK treatment for cancer in the later studies were 20-30%, because that there are to main shortcomings of Rosenberg's IL-2/LAK therapy. 1. Thaking too much blood from cancer patients. Using too high dosage of IL-2 which caused serious side effect and toxicity. We have made many improvements for the Rosenberg's IL-2/LAK treatment, and also got very good therapeatic results.

II) Protein engineering studies to generate new type rIL-2. There are three cysteins (Cys) in the IL-2 molecule. Thd 58 cys and 105 cys form a disulfide bond. The 125 cys is free which could cause the decrease of rIL-2 activity by the formation of 07 3 mismatched disulfide bond or by the formation of dimer between 125 Cys was replaced by Ser or Ala, using protein engineering method by substituting the Cys codon TGT with the Ser or Ala codon TCT or GCT. Two new type IL-2 gene were obtained and inserted into the expression vector to form expression plamids which yield 125 Ser-rIL-2 and 125Ala-rIL-2 in *E. coli* up to abotu 40% of total bacterial proteins. Using the purification method similar to natural rIL-2, 125 Ser-rIL-2 was purified to over 95% purity and crystallized. 125Ala-rIL-2 was found to have higher specificity and heat stablity which indicates a better new generation of rIL-2 were produced according to the information of IL-2 secondary structure and their activity were studied. IL-2 secondary structure and their activity were studied. IL-2 has four α- helix. When the fourth α-helix was destroyed by replacing 125 Cys with pro the activity of resulting molecule decreased. When two

Pro were introduced into the position of 125 and 127 of IL-2 to futher distroy the α-helix of IL-2, the activity was almost complely abolished. These studies may have both theoretical and practical meaning. It is hopeful to use these compounds as antitransplantation drugs. In addition, PEG-rIL-2 have been make and its half life t1/2 has been elongated. The study for getting IL-2 with higher acitivty, lower toxicity, lower antigenicity is an attractive problem.

III) High level expression of τ-Interferon (IFN-τ) total IFN-τ gene has been synthesized by phosphorimidine method in the collaboration of SIBAS and Fudan University and inserted into the high efficacy vector and expressed in *E. coli*. The yield of IFN-r amounted to 60-80% of the total bacterial proteins. Such a high level expression was hardly noted in literatures. Using a special procedure, IFN-τ can be isolated to more than 90% purity by the isolation of inclusion bodies and to 97% purity by subsequent sephacryl S-200 chramolography, the specific acitivity reached 2×10^7 international units/mg. In collaboration with the Secondary Military Medical University, we can produce more than 1.0g rIFN-τ has also been approved by the Chinese Ministry of public Health. In the genetic production of medical improtant protein, we have some superiority and have been applied to express TNF (tumor necrosis factor), SK (streptokinase) to an amount of more than 50% of the total host bacterial proteins.

Optimization of yeast expression system for the overproduction of anticoagulant hirudin

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We have studied the optimization of expression system for the overproduction of the potent thrombin-specific inhibitor, hirudin, using genetically engineered yeast, *Saccharomyces cerevisiae*. The expression and secretion of hirudin was directed by galactose-inducible promoter, GAL10 and mating factor α-pre-pro leader sequence (YEG α-HIR 5). The expression level of YEG α-HIR5 in shake flask culture was very low (2.37mg/*l*). Modification of expression vector and optimization of media and culture conditions greatly improved the expression level. Expression vector YEG α-HIR5 was modified to include GAL7 transcriptional terminator to the 3′ end of hirudin gene UYEG α-HIR 37) and TATA box of GAL1 promoter was deleted to block the transcription of GAL1 direction (YEG α-HIR525). These resulted in up to 40% increase in the expression level of hirudin per unit cell mass. Expression and secretion of hirudin seemed to be growth-dependent. Glycerol plus galactose was found to best support the growth and induction through the optimization study. The maximum hirudin level of 158 mg/*l* in the culture medium was obtained with fermentation in 4*l* scale under the optimized conditions.

Secretory High Expression of Human Epidermal Growth Factor in *Escherichia coli*

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Human epidermal growth factor (hEGF) is one of the most important growth factors being discovered and applied clinically for years. The intense potential as a therapeutic growth factor demonstrated by hEGF has make it a very attractive project in R & D for large scale production by recombinant DNA technology.

We have constructed a plasmid pAE-8 with a stretch of chemically synthesized hEGF coding sequence, and a strong promoter sequence. Upon

10 hrs fermentation of *E. coli* transformed by pAE-8, and expression level more than 307mg/*l* was obtained, and 90% of the expressed rhEGF was secreted into the culture medium.

The rhEGF was purified by a simple procedure and a purity higher than 90% in HPLC obtained easily. Amino acid sequencing data of the N-terminal 15 a.a. and the C-terminal 5 a.a. showed no difference between our hEGF and the native hEGF. Trypsin cleavage peptide mapping also gave the same pattern. The characteristic CD spectrum of rhEGF showed a feature with β-sheets but no α-helix, which was similar to that of the native EGF. The behavior of rhEGF in ELISA and RIA as well as its growth promoting activity to cultured cells are also not distinguishable from the native one. In summary, the pAE-8system was shown to be a new production system in *E. coli*. The fully secretory pathway made the rhEGF performed its native conformation and acquired full biological activity. As most of the rhEGF produced was appeared in the culture medium, the purification was very easy. Therefore, the pAE-8 system is a very promising system for large scale production of rhEGF.

Taranscriptional transactivcation of Human IGF-II Gene promoter 4 by Hepatitis B Virus Transactivator (X) in the formation of HCC

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Insulin like growth factor-II (IGF-II) is a highly mitogenic fetal growth factor suspected of regulating the growth of a wide spectrum of tissue via an autocrine or paracrine mode of action or both. Highly stady state levels of IFG-II RNA were detected in hepatocellular carcinoma (HCCs) arising from human and Woddchuck livers with persistent human and Woodchuck hepatitis virus infection. Four different promoters, preceding exon 1, 4, 4B and 5 have been identified in the human IGF-II gene. These promoters are activated in a tissue-specific and development specific manners. Promoters, preceding exon 1 is only usded for adult liver mRNAs, where as promotors preceding exon 4, 4B 5 are active in fetal liver tissues. But during the formation of HCC, promoter II, III and IV which are preceded by exon 4, 4B and 5 become actevated. We analyzed promoter IV in order to detect the activation mechanisms of IGF-II during the formation of HCC. We demonstrated that Rb can positively regulate transcription from the 4th promoter of IGF-II. Two copies of RCE (retinoblastoma controlling element) were identified in the 4th promoter of the IGF-II gene. We showed that the transcription factor Sp1 can bind to and stimulate transcription from the RCE motif. We also directly demonstrated that Rb positively regulated Sp1 transcriptional activity *in vivo*.

The hepatitis B virus-X gene product could be important in IGF-II activation because it is known to transactivate transcription from many viral and cellular gene promoter. Experimental results on HBV-X responsive element on IGF-II promoter 4 will be discussed.

Penicillin G Acylase

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Penicillin G is an industrially important enzyme, which hydrolyses penicillin G to phenylacetic acid and 6-amino penicillanic acid (6-APA).

The pac gene coding for penicillin G acylase is located on the chromosome of *E. coli* D816 and the pac gene was cloned into pBR322 by using EcoRI and PstI, the Pac^+ transformants were screened on NIPAB plates. The pac structural and regulatory genes was located on a 3,6 Kb Hind III fragment and the contiguous 2.7 Kb Hind III fragment enhanced the pac expression.

Several *E. coli* promoters were fused to pac gene, the highest expression was obtained with pac-2 promoter. The pac expression from Ppac-2 was regulated by temperature, oxygen, phenylacetic acid and cell growth rate. The pac regulatory gene was located within the pac structural gene.

A *E. clok* strain, *E. coli* A56, was selected as host for pac expression, because of high pac expression and low β-lactamase background. The yield of penicillin G acylase of *E. coli* A56 (pPA22) reached 7,000 IU/L, 20-fold higher than that of parent strain. A hollow fiber bioreactor was used to produce 6-APA in both pilot scale and commercial scale. The specific activity of the hollow fiber reactor was quite high, thus the high concentration of penicillin G could be converted to 6-APA within 2 h and the 6-APA could be directly crystalized. The average yield of 6-APA was 90.1% and the purity of 60APA was 98.7%. After 50 production cycles, the bioreactor remained 87% acylase activity.

Deyelopment of New Probiotics for Swine

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The acid and bile resistant *Lactobacillus* sp. originally isolated from animal wastes, was developed for probiotics to swine, The isolated *Lactobacillus* sp. was named as TSC 66, and its expreimental results were as follows.

1. Fifty percent of *Lactobacillus* sp. TSC 66 was survival after twenty minutes treatment in swine gastric juics adjusted to pH 2.0.

2. *Lactobacillus* sp. TSC 66 showed growth on the existence of 0.5% oxgall. It means *Lactobacillus* sp. TSC 66 be highly resistant to bile salt.

3. *Lactobacillus* sp. TSC 66 was safely passed through stomach and served as probiotics in the cecum from results of cannulae test.

4. The oral adminstration of *Lactobacillus* sp. TSC 66 can be reduced or replaced the significant amount of antibiotics use for promotion of animal growth.

Feasibility Studies for a Membrane Bioreactor to Intensity Biological Waste Water Treatment

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Current study is aimed to develop a membrane bioreactor system to intensify the waste water treatment process. Preliminary results indicates that it is possible to intensify the process. In this process, the biomass concentration can be controlled to a level appropriate to the occation where nearly complete reduction of BOD and limited active sludge propagation are needed. Compared with the advanced ICI deep shaft process for sewage treatment, current process has the advantage of smaller space (scale of bioreactor), the residence time of the sewage in the system is at least 3 times shorter than that of ICI treatment process.

The present process can be conducted under high loading ($kg/m^3/d$), high specific flow rate (Vw/Vr/d) and yet has the advantage of high BOD reduction percentage. Fouling of the membrane during operation could be partially overcome when backflushing with compressed air was applied.

Application of a fast response BOD measuring system to the present work has been done successfully. BOD sample could be determined within 30 min. There is a good linearity between the concentratin of a standard GGA soultion and relative current decrease. Thus, current BOD measuring

system can be potentially applied to the determination of any substrate, provided the substrate can be utilized readily by the microbe applied.

Secondary Metabolites Production by Extraction of Plant Cell Culture

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Effects of cell immobilization and in situ extraction on shikonin production in transformed plant cell and hairy root of *Lithospermum erythrorhizon* were studied. Shikonin production of transformed *L. erythrorhizon* increased with the enhanced oxygen supply, and in situ extration also increased sucrose consumptionand shikonin production. And in situ extraction at earlier stage significantly enhanced shikonin production. Sufficient oxygen supply and in situ extraction at earlier stage are reguired for the effective production of shikonin by the culture of transformed cells of *L. erythrorhizon*.

Plant hairy root cultures of *Lithospermum erythrorhizon* were carried out to produce shikonin derivatives by employing in situ extraction with n-hexadecane in shake flask and a bubble column bioreactor. Over 95% shikonin produced was recovered in the n-hexadecane layer. In flask cultures the maximum concentration of shikonin with n-hexadecane extraction was 3 times higher than that obtained without extraction. In the two phase bubble column reactor, 572.6 mg/L of shikonin and 15.6 g/L of dry cell mass were obtained after 54 days. Shikonin was produced at a constant level of 10.6 mg/L day during this period.

From the above results it can be concluded that hairy root culture with in situ extration is useful for shikonin production and effective recovery. Shikonin production can be further improved by using the two phase bubble column reactor as shown in this study. This technique may be useful for production of other useful secondary metabolites from plant hairy root culture and may also allow continuous operation.

Studies on Specfic Non-sterol Inhibitors of HMG-CoA Reductase

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It is well known that the major cause of death in the western countries and the big cities in china is coronary artery disease. A primary risk factor for the disease is known to be hypercholesterolemia. In humans 50% or more of the total body cholesterol is derived from de novo synthesis and elevated serum low-density lipoprotein (LDL), cholesterol is a mojor risk factor in the development of atherosclerosis and coronary artery disease. This fact has let to intensive efforts to discover inhibitors of cholesterol biosynthesis. The most suitable target for this inhibition is 30 hydroxy-3-methylglutaryl coenzyme in the pathway of cholesterol biosynthesis.

During the course of screening for new bioactive compounds from microorganisms, a simple, quick method for screening of cholesterol synthesis inhibitors was established in our Insititute. By means of this method, thousands of fungi culture filtrates have been examined for the inhibitory effect on cholesterol biosynthesis. It would be reported in this paper that several metabolites from three cultures of fungi, designed strains SIPI-8915, SIPI-8916, SIPI-8917, respectively, which were shown to be extremely potent competitive inhibitor of HMG-CoA reductase.

Bioactive compound 8915-I was isolated from the culture filtrate of strain SIPI-8915. It was identical with compactin (ML-236B) by a combination of physical techniques.

The structure of the bioactive compound 8917-

III was elucidated by mans of UV, IR, MS, NMR spectra, and was identical with that of bisdethiobis (methylthio) gliotoxin. Although it was a known compound, it was the first time to report about its inhibitoroy effect on the cholesterol biosynthesis.

The strain SIPI-8917 could be assumed as one strain of Zygo-mycotina, Zygomacetes, or Mucorales on the bais of Morphoilogical characteristics.

Further investigation for bioactive compounds isolated from the culture broth of Strain SIPI-8916 is in progress.

The Recorery of Poly(3-Hydroxybutyrate) from Alcaligenes Eutrophus

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Many different bacteria accumulated poly(3-hydroxybutrate), PHB, as an intracellular reseve material under untalanced growth conditions of excess carbon or energy source with limited other nutrients or growth factors.

A number of solvent extraction processes have been developed to recover PHB from biomass. For example, PHB can be extracted from bacterial cells with chlorform, methylene chloride, 1,2-dichloroethane, or propylene carbonate, However, these methods are either time-consuming or dangerous due to the explosive nature of the solvlents used. A simpler and faster procedure is the use of a differential digestion method employing sodium hypochlorite, Although simple and effective, this method has been avoided because it had been reported to cuse severe degradation of PHB. Recently, it was reported that by optimizing the conditions of sodium hypochlorite digestion and by balancing the ratio of hypochlorite to non-PHB biomass, PHB of 95% purity with a weight average molecular weight of 600,000 was recovered. The original molecular weight was 1,200,000.

To take advantages of both differential digestion and solvent extraction, we used dispersions of hypochlorite and chloroform. The rationale behind this method is as follows; PHB is hydrophobic, while cell powder is hydrophilic. When PHB is isolated from the cel by the action of hypochlorite, it will immediately migrate to the chloroform phase avoiding severe degradation Chloroform can, at least partially, protect the PHB molecules from further destructive action of the hypochlorite. The treatment with hypochlorite alone caused so severe degradation that the molecular weight decreased drastically with increasing hypochlorite concentration, However, using the dispersion, the degradation of PHB was markedly diminished owing to the shielding effect of chloroform, In this case, ws could obtain PHB of above 97% purity with a average molecular weight of 1,000,000 comparable to the original molecular weight of 1,200,000.

Studies on Specfic Non-sterol Inhibitors of HMG-CoA Reductase

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Biosurfactants occur in nature in great variety and perform important physiological functions. Presently they have become very important as there is wide range of applications, such as cosmic products, enhanced oil recovery, detergents, food and textiles, oil field chemicals and so on. The advantages of these biosurfactants over synthetic products are their biodegradability, nontoxic and relatively simple production by microbial fermentation.

Many kinds of biosurfactants were investigated by our laboratory, such as rhamnolipids, sophoroselipids, trehaloselipids, lipopolysacharide and other glycolipids. The glycolipids reduce remarka-

bly the interfacial tension between aqueous solution and n-hexadecane. Most biosurfactants show good emulsifying power, solubilization and some foaming ability.

A bacterial selected from soil smples was identified as a *Pseudomonas* sp. The strain was demonstrated that the more suitable medium contained rice bran oil (10%) as carbon source, $NaNO_3$ (0.5 %) as nitrogen source and yeast extract (0.05%) as organic nutrient. The initial pH of 6.5 proved to be optimal. Most of the rhamnolipids was produced in the late exponential phase of growth. After 72 hr cultured in a 10 L BioFlo IV fermenter, the yield of the glycolipids was 20 g/L. After purification by column and TLC, It was identified as rhamnolipid R_1 and rhamnolipid R_2 consisting of 1 or 2 molecules of rhamnose and B-hydroxycarbosylic acid residue.

Another glycolipid is sophoroselipids. Is was produced by a yeast *Torulopsos* sp. When it grown on carbohydrate, vegetable oil or water-insoluble alkanes. The structure of these glycolipies were also elucidation by means of IR, NMR, and MS. The main product is a lactonic sophoroselipids containing a mono-unsaturated 17-hydroxyoctadecanoic acid. The yield of sophoroselipids was 70 g/L when the yeast is cultures in a 10 L BioFo 1V fermentor for 96 hr. Using rice bran oil as the carbon source.

It was demonstrated that the CMC value of rhamnolipids (1×10^{-5} mole) was the lowest amone the above glycolipids. The reduce of the interfacial tension was also the lowest about 20 mN/m by rhamnolipids. For the purpose of enhanced oil recovery, many anti-ions were selected. When the ratio of rhamnolipis and sodium dodecyl sulfonate was 4000 : 1, it was the best formula to obtain the better results when its concentration was 150 ppm.

Finally we studied the dilute system of biosurfactant. Its interfacial tension was 2×10^{-3} mN/m and CMC value was 4×10^{-3} mole. The efficiency of driving residual oil was 40% in our expreimental equiments.

Immunological Enhancement of Ginseng Extracts in Mice Transplanted with Sarcoma 180 Tumor Cell

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The purpose of this study is to define the immunological enhancement of the ginseng extracts which are produced from Korea, Chian and America in transplanted mice with Sarcoma-180 tumor cells. We intentionally administered each ginseng fresh ginseng extract-fraction A,C,D) dissolved in saline at the dose of 0.2 m*l* a day (through 22 or 36 days) to mice which have been treated with the subcutaneous injection (dose 0.2 m*l*) of Sarcome-180 tumor cells)1×10^6 cells/m*l*). Immunological assay system was used as a chemotactic method and methods of macrophage migration inhibitory factor, natural killer and lymphokine activated killer cells activity were used. All data were assayed by using statistical methods (t test and p value). The results obtained were as follows;

1. The mice group treated with the ginseng extracts mentioned had the better leucocyte chemotactic effect, when tested with mice subcutaneously injected with Sarcoma-180, compared with the control group and, of the three, the Korean red ginseng was superior to others.

Tumor borne mice group treated with ginseng extracts the control group on the effect of MIF, but the mice group treated eith Korean red ginseng was superior to that treated with Chinese and American ginseng and the fraction C of Ko-

rean red ginseng had better effects.

3. In general, NK cell activity of mice group treated either the gineseng extract was better compared with the control group and, particularly, Korean red ginseng NK cell activity showed 7 times greater activiaty (5.1± 1.5) than the control group (0.7± 0.1).

4. IL-2 induced NK cell activity in ginseng-treated mice injected subcutaneously with Sarcoma 180 was better than the control group and the mice group treated with Korean ginseng was superior to the control group.

5. LAK cell activity of the mice treated with the ginseng after the injection of Sarcoma-180 was statistically more significant than the activity of the control group and the mice group treated with Korean red ginseng was statistically superior to the control group.

6. After the subcutaneous injection has been arranged, we administered the fraction to the treated mouse. The mass weight of tumor was decreased a little. Thus, the effect of ginseng has been visualized.

7. Blood cel distribution of tumor borne mouse treated with ginseng showed apparent change compared with the control group but the values of RBC, hemoglobin and hematocrit were increased.

According to these results, we can conclude that the treatment with ginseng would enhance the leucocyte chemotaxis ability and MIF effect as well as cytotoxicity effect of NK and LAK o) 3 cells. We found that NK cell and LAK cell had the ability to destroy the target cell and NK cell activity treated with IL-2 was apparently high.