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소 흉선 추출물의 암세포 증식 및 림프구에 미치는 영향

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Antitumor and Immunological Effects of Bovine Thymic Extract.

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Thymic extract showed antitumor effect to sarcoma mice with higher dose(200μg/mouse/day, i.p., 4weeks) but not with low dose(5μg/mouse/day, i.p., 6 weeks). Direct cytotoxicities were exhibited against sarcoma 180, L1210 and MOLT-4 by MTT assay. The spleen weight of mice were increased but the number of circulating lymphocytes were not increased after long-term(2 weeks) administration of thymic extract. Evaluating the mitogenesis by MTT assay, % absorbance of human lymphocytes was not increased by thymic extract. Cell cycle statistics of S phase and G_2/M phase was not increased in the presence of that by PI staining. The formation of rosette was induced, irrespectively of exposure time short-term(1 hour) and long-term(2 weeks). The population of mouse blood T-cell to bind Lyt2-antimonoclonal antibody and to L_3T_4 were increased after administration of thymic extract(2-200 μg/mouse/day).

From the above results, it is suggested that thymic extract exerts antitumor activity by stimulating T cells to differeniate in vivo but not in vitro.

INTRODUCTION

Mature and functional T-lymphocytes arise in the thymus. Their precusors, pre T-cells is originated in the bone marrow and settled in the cortex of the thymus. These cortical cells of the thymus of mouse are affected by thymic lymphocytopoietic factors and express three antigens called Lyt antigens and other.

The purpose of this experiment is to test antitumor activities of prepared bovine thymic extract as anticancer drug. Its antitumor activities were evaluated using MTT assay and cell cycle analysis by flow cytometry. And furthermore, it is intended to compare the biological characteristics of bovine thymic extract.

†본 논문에 관한 문의는 이 저자에게로.

EXPERIMENTAL

1. Used meterial and reagents

Concanvalin A(Con A) : Sigma Co., Propidium iodide(PI), RPMI 1640 3-(4, 5-dimethyl thiazol-2, 5-diphenyltetrazolium bromide(MTT.) : Sigma Co., Wright's solution : Mutopare Chem. Co., Lyt $_2$-FITC, L_2T_4-PE : Coulter. etc.

2. Preparation of thymic extract

Bovine thymus had been thawed and trimmed free of adipose tissue. 1 kg of thymus was homogenized in 1 liter of normal saline in a warming blender. The homogenate was heated with stirring to 80℃ in boiling water bath and freezed rapidly to -20℃ repeatedly. This solution was centrifuged at 800×g for 30 minutes at 4℃. The supernatant was filtered through Miracloth and subjected to ultrafiltrtion(M.W., 1,000-10,000 by molecular sieving method) at room temperature in an Amicon 8050. The portion of m.w. 1,000-10,000 was collected, concentrated by rotary evaporation under reduced pressure and dried by lyophilization. This material was stored and used routinely in this experiments.[1]

3. In vivo antitumor activity

In order to test in vivo cytotoxicity of thymic extract, male ICR mice were used. Sarcoma 180 cells were harvested by brief treatment with trypsin-EDTA and then maintained in abdominal cavities of mice by intraperitoneal transplantation of 2×10^6 cells/mouse of every 7 to 10 days. At first day, mice weighing 18-22 g were inoculated with 3×10^6 cells/mouse. Thymic extract(5μg/ml and 200μg/ml) or normal saline were injected intraperitoneally once a day for 4 weeks. The survival of treated sarcoma mice and the growth of tumor were observed carefully during 6 weeks. At sixth week, sarcoma mice were decapitated and their tumors and spleens were weighed.

4. Cell Lines

The following cell lines were used to test cytotoxicities of thymic extract. Molt-4 is a suspension culture derived from the peripheral blood of acute lymphoblastic leukemia patient. It is reportedly a stable T-cell leukemia.[2] Its doubling time is 20-22 hours.

L1210[3-4] is lymphocytic mouse leukemia and grows in suspension culture. The cells exhibit a doubling time of 8-10 hours and grow as stationary suspension cultures or spinner cultures. Sarcoma 180[5] was derived from Swiss Webster Sarcoma 180ascites. Its doubling time was 16-17 hours. These cell lines have been used extensively for routine screening programs of chemical agents and natural products for cytotoxic activity. These are also utilized in preliminary testing for antitumor activity in cancer chemotherapy screening studies.[6]

5. Preparation of lymphocytes

1) Human lymphocytes

Human lymphocytes were prepared from Isopaque-Ficoll(IF) density gradient centrifugation method which had been established by Buyum[7] : 10ml of heparinized blood was centrifuged for 10 minutes at 600×g. The leukocytes layer resting on top of erythrocytes pellet was removed(1 ml) and mixed with 1 ml of the colloidal iron suspension. The mixture was incubated in a shaking bath at 37℃ for 30 minutes. After 30 minutes, the cell suspension was mixed with equal volume of PBS and 4-6 ml was layered on top of 3ml IF solution and centrifuged. An almost pure suspension of lymphocytes was obtained from the interface.

2) Mouse lymphocytes(splenocytes)

The splenocytes of mice was prepared as described below.[8] The spleens of ICR mice were removed and put in a Petri dish containing a few ml of medium(Dulbecco's phosphate-buffered saline containing 2% bovine serum). The spleens were cut and gently disrupted. The large aggregated cells could be partly dissociated by repeatedly sucking up and down into a Pasteur pipette. Finally, the suspension

was filtered through a very loose pledget of absorbent cotton-wool and the absorbent was washed through with a few ml of fresh medium. Erythrocytes, contaminants in the splenocytes suspenison. were removed by treatment of 0.83 w/v% amonium chloride. Most of macrophages and granulocytes were also removed by drawing the cell suspension into a syringe which was been loosely prepared with glass wool. Dye exclusion tests(Trypan blue, 0.5%) were routinely performed on cell suspension. Prepared splenocytes suspensions contained approximately 90% viable cells.

6. Cell culture

The medium used in this experiment was RPMI 1640 medium supplemented with heat-inactivated fetal bovine serum(FBS, 10%), $NaHCO_3$(0.075%), glutamine(2mM) and antibiotics(100 units/ml of penicillin G and 100 μg/ml of streptomycin), and abbreviated to R10 medium.[9-10]

The preparedlymphocytes and cell lines were cultured in a humidified incubator maintained at 95% air : 5% CO_2 at 37℃

For the cytotoxic studies, cells used in all experiments were in the exponential phase of growth and maintained at 37℃ in growth medium.

7. MTT Assay

As an in vitro system for the screening of potency on cytotoxicities of thymic extract, MTT colorimetric assay was taken. This is depedent on the cellular reduction of MTT by the mitochondrial dehydrogenase of living cells to a blue MTT crystal of formazan which can be measured spectrophotometrically.[11-13] As shown in Fig.1, there is a good correlation between absorbance by MTT assay and the number of living cells. Percentage of control absorbance was considered to be the surviving fraction of the cells. Single cell of MOLT-4 and L1210 or Sarcoma 180 enumerated under a hemocytometer. The statistically counted equal number of cells were inoculated into 96 wells in 0.19ml of RI0 medium, to which 0.01ml of 20× concentration of thymic extract, or PBS was added. Following appropriate incubation of cell, 0.1mg(50μl of 2 mg/ml) MTT was added to each well and incubated at 37℃ for a further 4 hours. Plates were centrifuged at 450×g for 5 minutes on a plate holder and then the media was aspirated from plates. 150 μl of dimethyl sulfoxide was added to each well. The plates were placed on a shaker for 10 minutes to solubilize the bluecrystals and then read the absorbance immediately at 540 nm on a scanning multiwel spectrophotometer (enzyme-limked immunosorbent assay reader).

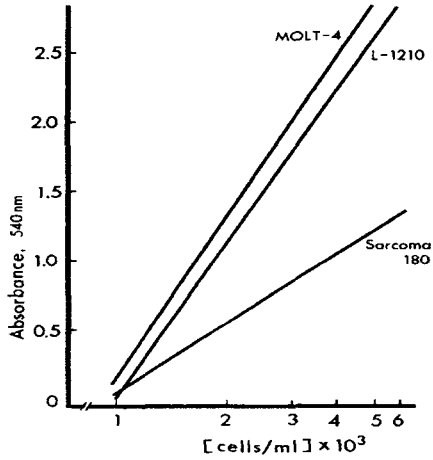


Fig. 1. Correlation between absorbance and the number of cells. MOLT-4, $y=3.90x-11.27$(x : logarithmic value, relation coefficient, $r=0.871$, the number of samples, $n=6$, probability, $p<0.05$) : L1210, $y=3.71x-10.94$ ($r=0.842$, $n=22$, $p<0.05$) : Sarcoma 180, $y=1.69x-4.84$($r=0.908$, $n=10$, $p<0.05$).

All data obtained were the mean of minimum of 3 wells. Decrease in percentage of control absorbance is considered to denote the cytotoxicity against the cells. For the proliferation of lymphocytes,

MTT assay had been intended to measure the change of the lymphocytes number following administration of thymic extract. However, it was almost impossible to detect the precise alteration in absorbance because minor change showed no significant difference statistically.

8. Wright's staining for microscopy

The change in white blood cells of mice pretreated with thymic extract for 2 weeks was examined by Wright's staining as follows.[14-15] 2 drops of the blood from each mouse was smeared on the slide for 1 minute. 2-3 volumes of buffered distilled water was added to the slide with care to avoid washing off the stain, and left for 5 min, and then washed off, and dried on the air. The white blood cells on the slide was enumerated under microscope(×400).

9. Cell cycle analysis by flow cytometry

The mitogenic activity of thymic extract to lymphocytes were estimated by cell cycle analysis using the modified technique described by Crissman and Steinkamp.[15] After 2-days culture, were fixed in 70 % ethanol for 1 hour and then stained with propidium iodide(0.1 mg/ml in citrate buffer solution, pH 7.6) in the presence of RNase(0.5 mg/ml).

Red DNA fluorescence of lymphocytes was measured using a FACS 440. 488nm line of an argon laser operating at a continuous output of 200 mW. Cell cycle analysis by DNA distribution was computed.

10. Evaluation of rosette formation

The splenocytes were obtained as described above for preparation of mouse lymphocytes. The cells were sospended in Hank's solution for counting and diluted to a concentration of 5×10^6 cells/ml. 20 ml of sheep blood was drained into 100 ml of Alservier's solution. The blood suspension was diluted with 5 volumes of Hank's solution and centrifuged at 500×g for 10 minutes. After the blood pellete was washed with Hank's solution, erythrocytes suspension was adjusted to a concentration of 1.8×10^{8} cells/ml in Hank's solution.

0.1 ml of splenocytes suspension(5×10^6/ml) and 0.05 ml of heatinactivated fetal bovine serum were added in separation tube containing 0.1 ml of sheep's erythrocytres suspension(1.8×10^8/ml). Following incubation on water bath of 37℃ for 5 minutes. this mixture was centrifuged at 200×g for 5 minutes and left at 4℃ for 2 hours. 0.1 ml of glutaraldehyde solution(0.8 w/v%) was mixed to each tube and stood of 20 minutes. After Giemsa staining, the cells was resuspended by gentle rotary agitation and the number of rosette forms of splenocytes on slide was counted under microscope(×400).[17]

11. Immunofluorescence staining for detection of surface antigens

This technique is based on the fact that cells having specific membrance antigens are detected by treating a cell population with monoclonal antibodies conjugated with fluorochromes.[18-20] It was used to examine whether the change in peripheral lymphocytes by long-term administration of thymic extract occurs or not and its relation to vivo antitumor activity.

The surface marker proteins to act as antigens vary in different member of the same species and different species. Among these, the mouse L3T4(m.w., 55KD) antigen and Leu 3a monoclonal antibody of human helper T lymphocytes is equvalent to the glycoprotein recognized by T_4, and anti-mouse Lyt2(m.w., 35KD) and Leu 2a of human cytotoxic/suppressor T lymphocytes by T_8[21-22]

Through simultaneous 2-color analysis by flow cytometer, the cells can be easily identified with recognization the determinants by one antibody conjugated FITC and the other antibody labeled with PE.[17] The flow cytometer. FACS 440, is equipped with standard filters to detect simultaneously emitted light at 530 nm(FLI, FITC, green) and at 585 nm(FL2, PE, orange).

At long-term administration the whole blood samples were obtained from mice to which had been administered with 5-200 μg/mouse/day of thymic extract and control mice with normal saline for 2 weeks, and erythrocytes were removed by treatment with hemolysis solution(ammonium chloride, 0.83 w/w %). Each 10 μl of FITC-and PE- conjugated reagents(anti-mouse Lyts-FITC and anti-mouse L 3T4-PE) was mixed in about 200 μl of samples suspended in PBS. After 30 minutes, these samples were washed with PBS twice and resuspended. Bindion to single leukocytes suspension(5×10^6 cells/ml) was analyzed by FACS440

Results and Discussion

1. In vivo antitumor activity of thymic extract

The antitumor activity of prepared thymic extract was evaluted using sarcoma mice which were inoculated in left groin subcutaneously. When administered with 5 μg/mouse/day, the growth of tumor was not to be affected, comparing with the control as shown in Table. 1. In higher dose of thymic extract of 200 μg/mouse/day, it was observed that at first tumorigenesis was similar to the control but the growth was rerarded and at last the tumor was

Table I. *Effect of thymic extract on tumor and spleen weight of 6 week-Sarcoma mice.*

group	injected dose (μg/mouse/day)	body weight (g)	tumor (g)	spleen (g)
Control,	vehicle*	27.5±2.3(7)	5.61±2.50	0.37±0.07
Thymic extract.	5	23.0±2.7(6)	5.94±2.22	0.36±0.07
	200	29.9±1.4(4)	0	N.D.

*, noraml saline, 0.1 ml/mouse/day.

N.D., not determined.

The number in parenthese means the number of sarcoma mice used in this experiment.

A

B

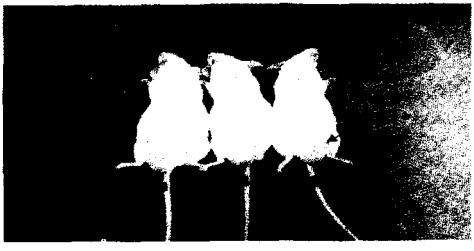


Photo. I. Presentation of antitumor effect of thymic extract.

A. Sarcoma mice of the control group which were injected with normal saline instead of thymic extract bore tumor 4 week after inoculation of Sarcoma 180(3×10^6/mouse, s.c. in left groin). "←" indicates the growing tumor.

B. Sarcoma of mice was disappeared and a spot of hairless remains in the left groin of mice. Mice were anesthetized with thiopental sodium(80 μg/kg).

disappered 4th week, remaining hairless spot, represented in Photo. 1. This result is in agreement with that neonatally thymectomized animals strikingly unhealthy and slow and apparent deterioration of cell-mediated immunity was progressed in thymectomized adult animal. The analytical findings are required to be added further by detailed investigation.

2. In vitro cytotoxicity of thymic extract

The direct cytotoxicity of thymic extract was estimated by comparing absorbance to the control which was cultivated without thymic extract. Fig. 2. shows that the absorbance of Sarcoma 180, LI210 or MPLT-4 cultured in medium containing thymic extract was not decreased dose-dependently It means that thymic extract exhibits no direct cytotoxicity. However, in the case of MOLT-4, the absorbance was lower than the others, irrespectively of administered dose.

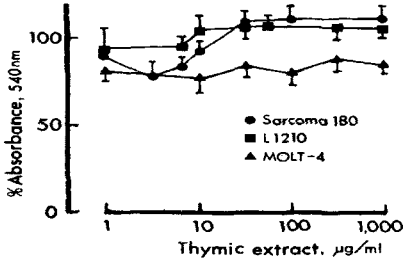


Fig. 2. Cytotoxicity of thymic extract on the proliferation of Sarcoma 180(●), LI210(■) and MOLT-4 (▲). Absorbance was measured by MTT assay during incubation of 3 days at various concentrations, and compared with that the control which cultivated in the absence of any lectin or thymic extract. Each value denotes the mean ± standard error.

3. Alterations in peripheral lymphocytes and spleen of mice after long-term administration of thymic extract.

Following injection of thymic extract of 5 μg/mouse/day for 2 weeks, whole blood was obtained and spleen was weighed. The leukocytes on each slide stained with Wright's staining solution was compared with the control to be injected normal saline instead of thymic extract. As shown in Table 2, there was the significant increase in weight of spleen but not in number of peripheral lymphocytes by does of 50 μg/mouse/day. Although the weight of spleens after long-time administration were increased, the amount of spleen per g of tissue was not changed by treatment of thymic extract.

Long-term treatment of thymic extract accelerated the enlargement of spleen significantly, but its enlargement did not provoke the increase in percentage of peripheral lymphocytes when tested with Wright's staining. Spleen is the second lymphoid tissue whose structure serves to trap antigens and lymphocytes. In this way, the cells capable of mounting an immune response to an antigen brought into close proximity with the antigen. Cell yields from spleen vary depending both on induced and

Table II. *The changes in spleen weight and circulating lymphocytes by long-term administration of thymic extract.*

	control group	thymic extract*
Spleen weight(g)	0.172±0.017(6)	0.283±0.028(5)'
(Splenocytes/g)	(1.51±0.10×10⁸)	(1.52±0.21×10⁸)
Lymphocytes[a] (% of WBC)	83.5±1.0(4)	82.3±0.9(4)

Thymic extract(5 μg/mouse/day, *) was administered intraperitoneally once a day for 2 weeks. The number in parentheses means the number of mice used in this experiment.

[a], Determined circulating lymphocytes by Wright's staining under microscopy.

', Significantly different from the control, $p<0.05$

background of immunization. So, the enlargement of spleen could result from increased level of immunity induced by thymic extract.

4. Evaluation of mitogenecity of thymic extract

To estimate the effect of thymic extract as a mitogen to lymphocytes, the absorbance of human lymphocytes cultured in medium containing thymic extract for 3 days was measured by MTT assay. Thymic extract did not increase it and not does dependently as shown in Fig. 3. It suggests that there are not mitogenic activity in vitro condition or rather direct cytotoxic effects to lymphocytes.

Cell cycle statistics of human and mouse lymphocytes cultivated for 2 days followed by PI staining showed that S phase and G2/M phase were increased in the presence of Con A, but not thymic extract(Fig. 4) Thymic extract did not show the chercteristics, which is to support the role as a stimulating factor to lymphocytes in this culture test.

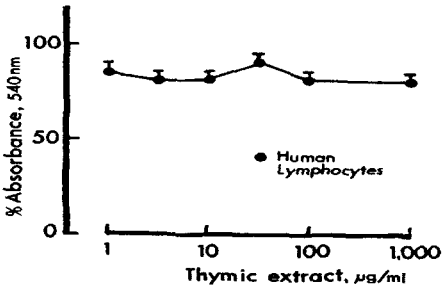


Fig. 3. Effects of thymic extract on the proliferation of human lymphocytes. Absorbance was measured by MTT assay after incubation for 3 days at various concentrations and compated with that the control. Each value denotes the mean ± standard error.

5. Rosette formation induced by thymic extract

The mature Y lymphocytes carry receptors which bind to determinants on the surface of sheep red blood, forming a rosette. Such "E" rosettes not only provide a convinient way to identify and count T lymphocytes but a way to separate T lymphocytes from B lymphocytes.

It was examined by counting E-rosettes under microscopy whether thymic extract can stimulate the in duction of T-cell or not as shown in photo. 2. Table 3. reveals that thymic extract is able to affect T-cells, even though with low concentration of 5 μg/mouse/day in which growing tumor was not depressed. When incubated with thymic extract for 1 hour, it was shown to be a also increased significantly.

The increased in rosette by treatment of thymic extract in vivo and in vitro means that thymic extract is able to induce T mouse and human lymphocytes to proliferate and differentiate.

Table III. *Increase in % rosette of mice splenocytes by administration of thymic extract.*

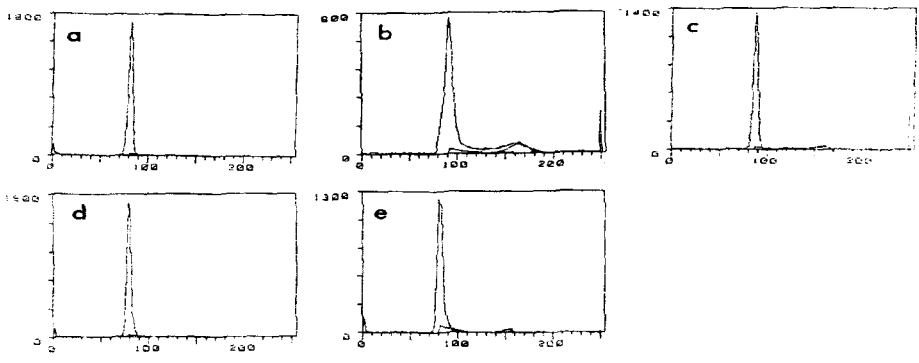
injected dose	control	thymic extract
once a day for 2 weeks(i.p.)		
Vehicle⁺	20.8±3.5	
5 μg/mouse		27.6±1.4*
20 μg/mouse		
incubation for 1 hr.		
Vehicle⁺⁺	6.1±1.4	
3 μg/ml		7.4±0.9
10 μg/ml		10.4±1.0*
30 μg/ml		8.8±1.1
100 μg/ml		10.3±1.1*

⁺, Normal saline, 0.1 ml/mousae/day

⁺⁺, Hank's solution.

*, Significantly different from the control, $p<0.05$.

A.



B.

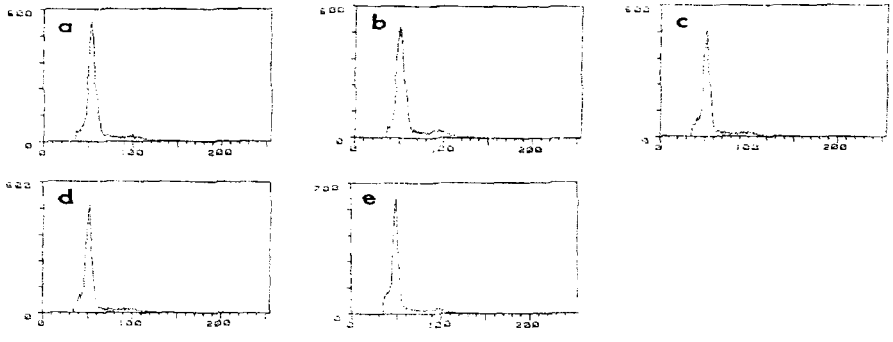


Fig. 4. DNA configuration of human(A) and mouse(B) lymphocytes cultivated in the presence of thymic extract. DNA content in lymphocytes was analyzed by flow cytometer(Becton/Dickinson, FACS440) as described in methods and materials. Effect of thymic extract(c : 10 μl/ml., d : 30 μl/ml., e : 100 μl/ml) was compared with the control(a) which was cultivated without any lectin or thymic extract and that in the presence of concanavalin A(b : 10 μg/ml). Vertical axis denotes cell number to be counted. Horizontal axis, relative DNA content.

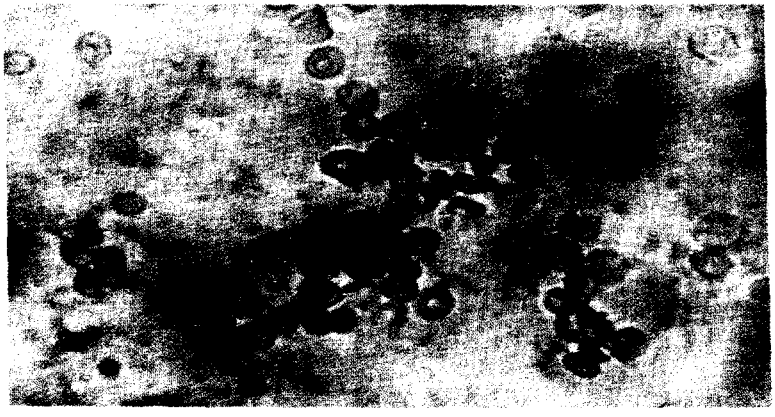


Photo. 2. Microphotographs showing typical "E"-Rosette(indicated) of a lymphocyte. The blood was drained from the mouse administered with thymic extract of 5 μg/mouse/day for 2 weeks and staind with Wright's staining solution. Microphotographs of cells were taken using an inverted microscope(IMT-2, Olympus) equipped with a camera(OM-10, Olympus).

6. Immunofluorescence staining for detection of T-lymphocytes surface antigens.

Further, these induction by thymic extract could be identified by the use of appropriate fluorescent anti-monoclonal antibodies. Lyt2 and L3T4 surface markers were detected using fluorescent anti-antibody binding method. Its measurement by flow cytometer represented as dot analysis(Fig. 5) and %gated(Table 4). Among the peripheral T lymphocytes of mice of long-term(2 weeks) administration with 5-200 μg/mouse/day of thymic extract, the population to bind Lyt2 antimonocional antibody and to L3T4 were increased significantly, suggesting the increase in numbers of activated T lymphocytes. This increase was more remarkable in %gated of lymphocytes containing Lyt2-surface antigen(cytotoxic/suppessor T cells) than L3T4(helper/inducer T cells).

From the above evidences, it could be summarized that thymic extract exerts in vivo antitumor effect via the potential stimulation of T lymphocytes.

Immunodeficiency states took place in thymectomized animals. In fact, thymus has been reported to produce the specific thymic hormones such as thymosin and thymopoietin 1 and 2 and thymic factors[23], where immature thymocytes can mature into functional T cells.[24] And, the utility of thymic extract tested in tumor bearing animals was found to be derived from the enhanced immunologic functions of mature T lymphocytes by thymic extract. This can be hopeful as an antitumor agent. furthermore, it its required to measure how much thymic extract contains the actual substances to suppress tumor.

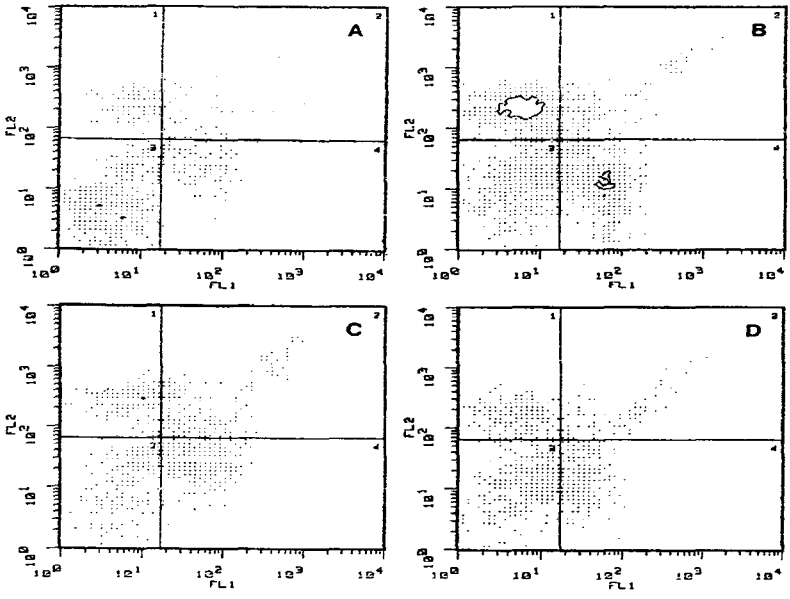


Fig. 5. Dot analysis for T lymphocyte surface antigen by two-color analysis. FL1 versus FL2 dot analysis displays while flow cytometer is adjusted for two-color analysis. Four distinguishable population were divided by dashed line which separate negative from positive(1 : FL2 positive, 2 : positive both FL1 and FL2, 3 : negative both FL1 and FL2, 4 : FL1 positive). The lymphocytes were obtained from mice which thymic extract was administered to for 2 weeks(A : control in the absence of thymic extract, B : 5 μl/mouse/day, c : 50, d : 200). % Gated of these analyses were expresented in Table 4.

Table Ⅳ. *%Gated of two-color analysis of mouse circulating T-cell surface antigen using anti-mouse monoclonal antibodies after long-term (2 weeks) administration of thymic extract.*

injected dose (μg/mouse/day)	anti-mouse monoclonal antibodies			
	L3T4	Lyt2	L3T4/Lyt2	unbound[#]
Vehicle[-]	23.4±9.5	16.5±4.4	10.3±6.4	49.7±10.3
5	27.0±6.5	33.0±3.5*	15.3±5.5	24.6± 2.5
50	30.6±3.6	24.3±7.8	5.1±1.1	40.1± 8.6
200	19.8±7.1	24.5±2.0*	21.6±3.7*	34.2± 4.4

[#], Unbound with neither L3T4 nor Lyt2.

[-], Normal saline 0.1 ml/mouse/day.

*, Significantly different from the control(vehicle injected), $p<0.05$.

Summary

Thymic extract were prepared for testing their antitumor and immunological effects. Thymic extract showed antitumor effect to sarcoma mice with higher dose(200 μg/mouse/day, i.p., 4 weeks) but not with low dose(5 μg/mouse/day, i.p., 6 weeks). Thymic extract did not exhibit direct cytotoxicities against Sarcoma 180, LI210 and MOLT-4 by MTT assay. The spleen weights and the number of splenocytes of mice increased, but that of circulating lymphocytes did not after long-term(2 weeks) administration of thymic extract. Evaluating the mitogenesis to lymphocytes by MTT assay, % absorbance of human lymphocytes did not increase in the presence of thymic extract. Cell cycle statistics of S phase and G2/M phase was increased in the presence of concanavalin a(10 μg/ml), but not thymic extract(10-100 μl/ml) by PI staining. Thymic extract induced the formation of resette, irrespectively of exposure time, short-term(1 hour) and long-time (2 week). The populations of mouse blood T-cell to bind Lyt2-antimonoclonal antibody and to L3T4 were increased after administration of thymi extract (5-200 μg/mouse/day).

From the above results, it is concluded that thymic extract exerts antitumor activity by stimulating T cells to differentiate in vivo but in vitro.

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