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# Different Immunosuppression on Specific Anti-tumor and Non-specific Cellular Immunity of Tumor-bearing Mice Following Tumor Growth

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## Abstract

Specific anti-tumor cellular immunity was assessed with indirect macrophage migration inhibition test and Winn assay. Non-specific lymphoproliferative responses to mitogens were also studied. In the mice bearing SC42 tumor, specific *in vivo* anti-tumor immunity was elicited at early stage of tumor growth, when the spleen cells showed positive macrophage migration factor (MIF) activity. The lymphoproliferative responses of the spleen cells were suppressed. Removal of tumor not only recovered these suppressed immunities but also gave tumor neutralizing activity to the spleen cells in Winn assay. Passage through glass wool column restored lymphoproliferative responses of tumor-bearing spleen cells to normal spleen cell level. Their MIF activity became positive after this treatment. The spleen cells of the mice bearing large tumor suppressed lymphoproliferative responses of normal spleen cells in co-culture assay. In contrast, they did not suppress positive MIF activity of tumor-removed spleen cells when they were co-cultured. These results suggested that there would be different immunosuppression on specific anti-tumor immunity and non-specific lymphoproliferative responses to mitogens in tumor-bearing mice.

## Introduction

It is widely accepted that immunity of tumor-bearing host deteriorates as a tumor grows<sup>5,6,8</sup>. The mechanism of this deterioration has been proposed by many investigators. It was reported that sera from cancer patients blocked cell-mediated tumor immunity<sup>10</sup>. There were many investigations which showed the suppressive effects of lymphocytes on either specific or non-specific immunity<sup>27,32</sup>. It has been reported that macrophage plays a role in the suppression of cellular immunity of tumor-bearing hosts<sup>2,16</sup>. The relationship between these different mechanisms of suppression in a certain tumor-host system is important in the evaluation of immune

Key words: Macrophage migration inhibition factor (MIF), Winn assay, Lymphoproliferative response, Tumor immunity, Immunosuppression.

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status of tumor-bearing hosts and cancer immunotherapy. In this report, immunosuppression on specific anti-tumor immunity of murine spleen cells assayed by macrophage migration inhibition test and on their non-specific lymphoproliferative responses to mitogens following tumor growth and removal of tumor were studied.

### Materials and Methods

#### Mice and tumor.

All experiments were performed with 6 to 10 week-old male DS mice which had been established as syngeneic mice from dd mice<sup>36)</sup> and kindly offered by the Aburabi Laboratories of Shionogi Pharm. Co. Shiga, Japan. Shionogi carcinoma 42 (SC42)<sup>22)</sup>, spontaneous mammary carcinoma of DS mice, was obtained from Dr. Kenji Yamaguchi, Institute of Shionogi Pharm. Co., Osaka, Japan and maintained by serial passages in DS male mice.

#### Transplantation and removal of tumor.

Tumor masses were minced and treated with 0.25% trypsin (Difco) in phosphate buffered saline (PBS) for 10 min at 37°C. Single cell suspension was obtained by passage through cotton gauge. These tumor cell suspension had a viability of 85 to 95% measured by trypan blue dye exclusion. The cell suspension containing  $2 \times 10^5$  or  $1 \times 10^6$  viable cells was injected into the footpad of hindleg. The thickness of footpad was measured periodically as tumor size, or tumor weight was measured by weighing the amputated legs. Removal of tumor was performed by amputation of hindleg with tumor below the knee.

#### Spleen cell preparation and partial removal of glass adherent cells.

Spleens were obtained aseptically, minced and passed through #100 stainless mesh. The spleens from five mice of one group were pooled in MIF assay, Winn assay and lymphoproliferative response assay. Erythrocytes were ruptured by incubation with 0.83%  $\text{NH}_4\text{Cl}$  in Tris buffer for 3 min at 37°C. Cell debris and clumps were allowed to settle by gravity for 5 min. The dissociated cells were washed with Hanks' balanced salt solution (HBSS) and viability was measured with trypan blue dye exclusion. They had a viability of more than 90%.

Glass wool was packed into 10 ml glass syringe and autoclaved. The glass wool column was washed with HBSS and RPMI1640 supplemented with 10% fetal calf serum (FCS, Micro Biomedics). The spleen cells suspended in RPMI 1640 with 10% FCS were applied to the columns and washed out with the same medium. The percentage of peroxidase positive cells decreased from 7.4% to 6.7% in normal spleen cell, from 12.2% to 6.5% in tumor-removed spleen cell and from 21.1% to 16.2% in tumor-bearing spleen cell.

#### Antigen.

The solubilization of tumor extract was performed by ultrasonication with the method of Haughton<sup>9)</sup> which was applied to H-2 antigen solubilization. The tumor cells were suspended in HBSS and cell concentration was adjusted to  $1 \times 10^8$ /ml. The ultrasonic cell breakage was performed for 90 sec with an ultrasonic disruptor (Tomy Seiko). The supernatant after centri-

fugation 10,000 rpm for 60 min was sterilized by filtration through 0.45  $\mu$  Milipore filter. The protein concentration was determined by Lowry's method<sup>18)</sup> and adjusted to 10 mg/ml. The supernatant was stored at  $-20^{\circ}\text{C}$  until use.

Winn assay.

In vivo cytotoxic ability of the spleen cells was assessed by Winn assay<sup>35)</sup>. Twenty million of the spleen cells mixed with  $2 \times 10^5$  of the tumor cells were inoculated in footpad of normal DS mice. Whether the tumor cells were rejected or not was determined five weeks after inoculation.

Indirect macrophage migration inhibition test.

a) First culture: Spleen cells were suspended in RPMI 1640 supplemented with 100  $\mu\text{g}/\text{ml}$  of gentamycin and adjusted to  $1 \times 10^7/\text{ml}$ . One ml of spleen cell suspension was incubated with or without 0.1 ml of the antigen in 5%  $\text{CO}_2$  in air at  $37^{\circ}\text{C}$  for 24 hr. The spleen cell suspension incubated without antigen was supplemented with 0.1 ml of the antigen at the end of incubation as control. They were centrifuged at 3,000 rpm for 20 min and the supernatants were stored at  $-20^{\circ}\text{C}$  until second culture.

b) Second culture: The agarose droplet method described by Harrington<sup>7)</sup> was used in this assay. The peritoneal exudate cells (PEC) of normal DS mice induced by mineral oil (Bayol F, Wako Pure Chemical Indust.) were packed in 0.2% agarose (Sea Plaque Agarose, Marine Colloids). Two microliter of agarose droplet was placed at the bottom of each well of Micro Testplate II (Nunc) by the microdispenser. After the settling down of the droplets, 0.1 ml of supernatants of first culture supplemented with 10% FCS was poured into each well and incubated for 24 hrs at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air. Then they were photographed under microscope.

The distance of migration of PEC from the edge of the agarose droplet in four directions was measured. Assay was quadruplicated and migration index (MI) was calculated as follows.

$$\text{MI} = \frac{\text{mean distance of migration of PEC in the supernatant cultured with the antigen}}{\text{mean distance of migration of PEC in the supernatant cultured without the antigen}} \times 100$$

Less than 86 was regarded as positive.

Lymphoproliferative responses to mitogens.

The spleen cells were suspended in RPMI 1640 supplemented with 10% FCS at cell concentration of  $5 \times 10^6/\text{ml}$ . A 0.2 ml aliquot of the cell suspension was incubated in a well of Micro Testplate II with or without a mitogen. As mitogens Con A (Wako Pure Chemical Indust.) and LPS (Difco) were added 1  $\mu\text{g}/\text{well}$  and 10  $\mu\text{g}/\text{well}$ , respectively. The plates were incubated for 48 hrs at  $37^{\circ}\text{C}$  in 5%  $\text{CO}_2$  in air and then, 0.25  $\mu\text{Ci}$  of  $^3\text{H}$ -thymidine (New England Nuclear) was added to each well. After 17 hr incubation, the cells were harvested with Cell Harvester (Mark II, Wakenyaku) and  $^3\text{H}$ -thymidine uptake was measured with a liquid scintillation counter (ISOCAP 300, Nuclear Chicago). Assay was triplicated and lymphoproliferative responses to mitogens were assessed as follows:

$\Delta\text{cpm} = \text{cpm with mitogen} - \text{cpm without mitogen}$

Statistical analysis.

The  $\chi^2$  test was used to evaluate the significance of differences between groups in in vivo anti-tumor immunity and Winn assay.

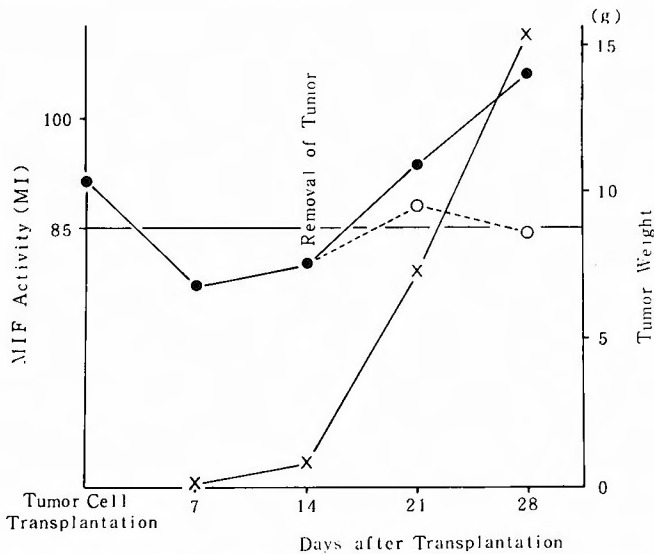
### Results

Macrophage migration inhibition factor (MIF) activity of spleen cells following tumor growth and removal of tumor.

The specific anti-tumor cellular immunity was assayed with an indirect macrophage migration inhibition test. About one week after transplantation of  $1 \times 10^6$  SC 42 tumor cells in footpad tumors became palpable. The spleen cells of the mice on day 7 showed positive MIF activity to the antigen solubilized by ultrasonication (Fig. 1). When tumors grew gradually, the spleen cells of the mice on day 14 still had positive MIF activity. As the tumors rapidly enlarged thereafter, the spleen cells on day 21 and day 28 lost positive MIF activity. The spleen cells of the mice whose tumors had been removed on day 14 recovered positive MIF activity on day 28. The normal spleen cells did not show positive MIF activity to the antigen.

Lymphoproliferative responses of spleen cells to mitogens following tumor growth and removal of tumor.

Lymphoproliferative responses of the tumor-bearing spleen cells to Con A and LPS had already decreased on day 7 after tumor cell transplantation, when their MIF activity became positive (Fig. 2). The response to Con A of spleen cells on day 14 decreased to half that of normal spleen cells and the response to LPS was almost completely depressed when their MIF



**Fig. 1.** MIF activity of spleen cells following tumor growth and removal of tumor. MI less than 86 was regarded as positive MIF activity. (●) MI of tumor-bearing spleen cells, (○) MI of tumor-removed spleen cells and (x) tumor weight.

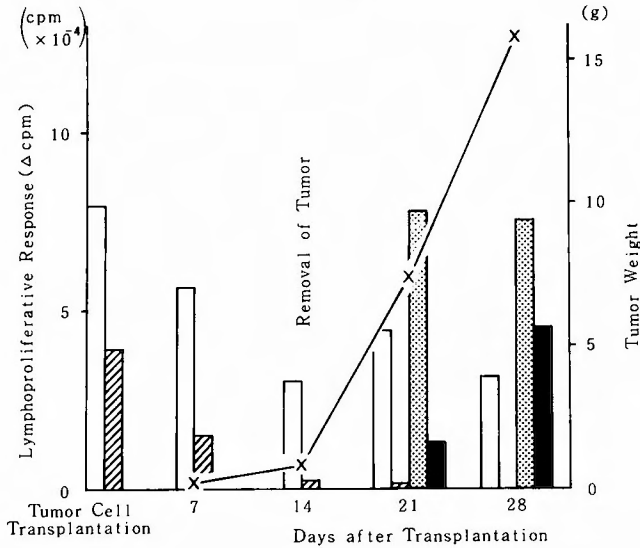


Fig. 2. Lymphoproliferative responses of spleen cells to mitogens following tumor growth and removal of tumor.  $\Delta\text{cpm} = \text{cpm with mitogen} - \text{cpm without mitogen}$ . (Open bar: response to Con A of tumor-bearing spleen cells, striped bar: response to LPS of tumor-bearing spleen cells, dotted bar: response to Con A of tumor-removed spleen cells and closed bar: response to LPS of tumor-removed spleen cells. (x) tumor weight.

activity was still positive. When tumors grew rapidly thereafter and MIF activity of the tumor-bearing spleen cells became negative, their lymphoproliferative responses to Con A on day 21 and day 28 were preserved. Removal of tumor on day 14 not only restored mitogen response to both Con A and LPS but also recovered MIF activity.

#### In vivo anti-tumor immunity of mice following tumor growth.

In order to define in vivo anti-tumor immunity of tumor-bearing mice, the mice were re-challenged with tumor cells on day 28 after tumors were removed on day 7, 14, 21 or 28. The mice which received removal of tumor on day 7 or 14 rejected tumor cell rechallenge. On the other hand, those which received it on day 21 or 28 allowed tumor growth (Table 1). This difference was statistically significant ( $p < 0.01$ ). When  $2 \times 10^5$  of tumor cells were transplanted, tumors grew more slowly and 4 of 7 mice whose tumors were removed on day 7 admitted the growth of rechallenged tumor cells. All of the mice whose tumors were removed on day 14 or 21 rejected rechallenged tumors. These results suggested that tumor transplantation elicited anti-tumor immunity in transplanted mice and that some tumor mass would be necessary for the immunity to be established. Moreover, large tumor suppressed this immunity.

#### Anti-tumor activity of spleen cells in Winn assay.

To elucidate competent immunity to resist tumor cell rechallenge, Winn assay of spleen cells was performed. One of nine mice to which the spleen cells of tumor-removed mice were transferred with the tumor cells allowed tumor growth. Most of mice to which the spleen cells of the mice bearing large tumor or of normal mice were transferred with tumor cells allowed tumor

**Table 1.** Anti-tumor Immunity of Mice Following Tumor Growth

tumor cell transplant <sup>a</sup>	removal of tumor on day <sup>b</sup>	tumor cell challenge <sup>c</sup>	rejected/total <sup>d</sup>
1×10 <sup>6</sup>	7 or 14	1×10 <sup>6</sup>	6/7 <sup>e</sup>
1×10 <sup>6</sup>	21 or 28	1×10 <sup>6</sup>	0/7 <sup>e</sup>
—	—	1×10 <sup>6</sup>	1/5
2×10 <sup>5</sup>	7	2×10 <sup>5</sup>	3/7
2×10 <sup>5</sup>	14	2×10 <sup>5</sup>	6/6
2×10 <sup>5</sup>	21	2×10 <sup>5</sup>	4/4
—	—	2×10 <sup>5</sup>	2/6

a: Tumor cells were transplanted into footpad of foreleg on day 0. b: Tumors were removed by amputation of foreleg. c: Tumor cells were challenged into footpad of hindleg on day 28. d: The number of the mice rejected tumor cell challenge was determined on day 56. e:  $p < 0.01$ .

growth (Table 2). The difference between tumor-removed spleen cells and normal spleen cells was statistically significant ( $p < 0.01$ ). This anti-tumor activity of the spleen cells in Winn assay was well correlated with their MIF activity.

Effect of removal of glass wool adherent cells on MIF activity and lymphoproliferative responses.

As tumors grew, spleens were enlarged. Percentage of peroxidase positive cells in spleen cells increased following tumor growth. This suggested that non-lymphocytic cells were involved in the depression of MIF activity and lymphoproliferative responses of tumor-bearing spleen cells. The percentage of peroxidase positive cells in tumor-bearing spleen cells was decreased after passage through glass wool column. While MIF activity of the whole spleen cells of the mice bearing large tumor was negative, the glass wool column passed cells of them showed positive MIF activity (Table 3). Both whole cells and glass wool column passed cells in the spleen of the mice bearing small tumor showed positive MIF activity. Neither the whole cells nor the glass wool column passed cells in the normal spleen showed positive MIF activity. Although MIF activity of whole spleen cells of the tumor-removed mice was positive, the cells passed through glass wool column lost the positive MIF activity.

Lymphoproliferative responses of tumor-bearing spleen cells to Con A were fully recovered by passage through the column and those to LPS were recovered partially. This treatment had little effects on normal and tumor-removed spleen cells (Table 4).

**Table 2.** Anti-tumor Immunity of Spleen Cells in Winn Assay

spleen cells <sup>a</sup>	tumor cells	rejected/total <sup>b</sup>
normal 2×10 <sup>7</sup>	2×10 <sup>5</sup>	1/9 <sup>c</sup>
tumor-removed 2×10 <sup>7</sup>	2×10 <sup>5</sup>	8/9 <sup>cd</sup>
tumor-bearing 2×10 <sup>7</sup>	2×10 <sup>5</sup>	1/5 <sup>d</sup>
—	2×10 <sup>5</sup>	1/5

a: Tumor-removed spleen cells were obtained on day 28 from the mice whose tumors had been removed on day 14. Tumor-bearing spleen cells were from the mice on day 28 after tumor cell transplantation. b: The number of tumor rejected mice was assessed on day 35. c:  $p < 0.01$ . d:  $p < 0.05$ .

**Table 3.** Effect of Passage through Glass Wool Column on MIF Activity

spleen cells	MIF activity (MI)	
	whole	glass wool column passed
normal	91	97
small tumor-bearing <sup>a</sup>	80 <sup>d</sup>	85 <sup>d</sup>
large tumor-bearing <sup>b</sup>	106	79 <sup>d</sup>
tumor-removed <sup>c</sup>	84 <sup>d</sup>	105

a: The spleen cells were obtained from the mice on day 14 after tumor cell transplantation. b: the spleen cells from the mice on day 28. c: The spleen cells were from the mice on day 28 whose tumors were removed on day 14. d: MI less than 86 was regarded as positive MIF activity

**Table 4.** Effect of Passage through Glass Wool Column on Lymphoproliferative Responses

spleen cells <sup>a</sup>	Con A		LPS	
	whole	passed <sup>b</sup>	whole	passed <sup>b</sup>
normal	79584 <sup>c</sup>	93088	38606	62810
small tumor-bearing	30235	74176	1789	19798
large tumor-bearing	29813	93474	-374	13296
tumor-removed	73706	51905	44027	45963

a: Spleen cells were same as Table 3. b: glass wool column passed cells. c:  $\Delta\text{cpm} = \text{cpm with mitogen} - \text{cpm without mitogen}$ .

#### Suppressive activity of tumor-bearing spleen cells.

Recovery of MIF activity and lymphoproliferative responses by passage through glass wool column suspected the existence of suppressor cells in tumor-bearing spleen cells. To certify this suspicion, co-culture of spleen cells in vitro assay was done. The spleen cells of the mice bearing large tumor suppressed lymphoproliferative responses to both Con A and LPS of the normal spleen cells co-cultured (Table 5). On the other hand, they did not suppress positive MIF activity of the tumor-removed spleen cells (Table 6). These results suggested that mechanisms of suppression of specific anti-tumor immunity and nonspecific immunity might be different.

**Table 5.** Effect of Co-culture with Tumor-bearing Spleen Cells on Lymphoproliferative Responses of Normal Spleen Cells

spleen cells <sup>a</sup>	Con A	LPS
normal	65934 <sup>b</sup>	47481
tumor-bearing	15260	6115
normal + tumor-bearing <sup>c</sup>	24111	18238

a: Tumor-bearing spleen cells were obtained from the mice on day 28 after tumor cell transplantation. b:  $\Delta\text{cpm} = \text{cpm with mitogen} - \text{cpm without mitogen}$ . c: Normal spleen cells and tumor-bearing spleen cells were mixed at 1:1 ratio.



**Table 6.** Effect of Co-culture with Tumor-bearing Spleen Cells on MIF Activity of Tumor-removed Spleen Cells

spleen cells <sup>a</sup>	MIF activity (MI)
normal	110
tumor-bearing	102
tumor-removed	82 <sup>b</sup>
tumor-removed + normal <sup>c</sup>	80 <sup>b</sup>
tumor-removed + tumor-bearing <sup>c</sup>	81 <sup>b</sup>

a: Tumor-bearing spleen cells were obtained from the mice on day 28 after tumor cell transplantation and tumor-removed spleen cells from those on day 28 whose tumors were removed on day 14. b: MI less than 86 was regarded as positive MIF activity. c: Co-culture was performed at 1:1 ratio.

### Discussion

Macrophage migration inhibition test has been one of the reliable methods to assess cellular immunities *in vitro* concerned with delayed type hypersensitivity<sup>1,4,15,28,30</sup>. This method has also been applied to assay specific anti-tumor immunity of tumor-bearing hosts and to detect tumor-specific antigens<sup>3,20,29</sup>. The tumor extract solubilized with frozen and thaw<sup>14</sup>, with 3 M KCl<sup>17,21,31</sup>, with Nonidet P-40<sup>23</sup>, with papain<sup>19</sup> and irradiated tumor cells<sup>33</sup> were used as antigens for this assay. Cell extract solubilized with ultrasonication possessed H-2 antigen as measured by heamoagglutination inhibition<sup>9</sup>. SC 42 tumor cell extract solubilized with this method was used as an antigen for this assay assessing specific anti-tumor immunity of murine spleen cells in this study. MIF activity of the tumor-bearing spleen cells became positive on day 7 and 14 after tumor transplantation, while the normal spleen cells showed negative MIF activity. As the tumor grew rapidly thereafter, MIF activity of the spleen cells became negative. These results were also well correlated with *in vivo* anti-tumor immunity of the mice, that is, the mice whose tumor removed on day 7 or 14 after tumor transplantation rejected tumor cell challenge, while those whose tumor removed on day 21 or 28 allowed tumor growth. This indicated that the tumor-bearing mice obtained specific anti-tumor activity as the tumor settled in the mice and as the tumor grew rapidly thereafter the mice lost the activity, and that the anti-tumor immunity would be assessed with macrophage migration inhibition test.

Decrease of lymphoproliferative response in tumor-bearing hosts were reported by many investigators<sup>2,6,16</sup>. The lymphoproliferative response of the tumor-bearing spleen cells to Con A and LPS decreased from early stage of the tumor growth when the spleen cells showed positive MIF activity (Figure 1 and 2). After removal of tumor, the lymphoproliferative response to Con A and LPS reverted to the normal level. Positive MIF activity which would be lost with progressive tumor growth reappeared in tumor-removed spleen cells. Moreover, these cells possessed strong tumor neutralizing activity in Winn assay, while neither the spleen cells of large tumor bearer nor the normal spleen cells did. These results indicated that removal of tumor not only recovered nonspecific lymphoproliferative response to mitogens but also restored and strengthened specific anti-tumor immunity, confirming immunological effectiveness of reduction

surgery in cancer therapy.

KIRCHNER et al.<sup>12)</sup> reported that spleen from Moloney sarcoma virus tumor-bearing mice contained four times the number of mononuclear cells and the decreased response to PHA of these spleen cells restored by purification with rayon column or by removal of phagocytic cells by an iron/magnet technique, and they suggested that macrophage/monocyte series played a role in this immunosuppressive mechanism. PADARATHSINGH et al.<sup>24)</sup> also observed that esterase positive cells or Fc receptors positive cells increased in tumor-bearing spleen with progressive tumor growth and that treatment of these spleen cells by passing through Sephadex G-10 columns or by incubation on plastic surfaces to remove adherent cells restored lymphoproliferative responses. SC 42 tumor-bearing spleen contained increased peroxidase positive cells with tumor growth. Removal of glass adherent cells by passing through glass wool columns not only restored the lymphoproliferative responses to mitogens but also recovered positive MIF activity (Table 3 and 4). From these results it was suggested that in tumor-bearing mice the glass adherent cells not only suppressed non-specific lymphoproliferative responses but also regulated specific anti-tumor immunity.

Co-culture with the normal spleen cells confirmed the immunosuppressive activity of macrophage/monocyte series in tumor-bearing spleens<sup>13,25,26,34)</sup> and the peripheral blood of cancer patients<sup>37)</sup>. In the present study, the spleen cells of large tumor bearer suppressed the lymphoproliferative response to Con A and LPS of co-cultured normal spleen cells, concur with the results of other investigators. On the other hand, they did not suppress the positive MIF activity of the tumor-removed spleen cells when they were co-cultured. The suppressive mechanisms of the specific anti-tumor immunity assessed with macrophage migration inhibition test and its competent cells are now under investigation at our laboratory. Moreover, the fact that though suppressed, the host bearing large tumor still had specific anti-tumor immunity would be a clue of specific immunotherapy for cancer patients. Our recent experiments showed that spleen cells of small tumor bearer had weak tumor neutralizing activity in the Winn assay, which was strengthened by removal of plastic adherent cells. The anti-tumor activity of these cells were augmented by culture with the tumor extract solubilized with ultrasonication and these cells were proliferated by culture with T cell growth factor. When these augmented and proliferated cells were transferred to the tumor-bearing mice locally, they inhibited the tumor growth<sup>11)</sup>.

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## 和文抄録

# 腫瘍増殖にともなう免疫抑制に関する実験的研究

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同系の DS マウスと同系腫瘍 SC42 を用いて, 腫瘍増殖にともなう特異的抗腫瘍免疫能を脾細胞のマクロファージ遊走阻止試験および腫瘍中和試験にて検索し, 非特異的免疫能を各種 mitogen に対する幼若化反応を用いて検索した. 腫瘍移植後早期に宿主は腫瘍抵抗性を獲得し, その脾細胞は腫瘍抗原に対するマクロファージ遊走阻止因子活性陽性を示した. 一方, 幼若化反応はこの時期から低下した. 腫瘍が増殖するに従って, これらのいずれの免疫能も抑制されたが, 腫瘍切除によりいずれも回復した. そして, 腫瘍切除を受けた脾細胞は腫瘍中和試験において強い抗腫瘍能を示した. このことは, 癌外科における reduction surgery の腫瘍免疫の面からの意義が示されたと考えられる. 腫瘍を持った宿主の脾細胞の抑制された免疫能

はガラス線維カラムを通すことによりいずれも回復した. また, 正常脾細胞と腫瘍を持った脾細胞を混合培養すると, 正常脾細胞の幼若化反応は抑制されたが, 腫瘍切除を受けた脾細胞のマクロファージ遊走阻止因子活性は腫瘍を持った脾細胞との混合培養によっても抑制を受けなかった. これらのことは腫瘍増殖にともなう特異的抗腫瘍免疫能と非特異的免疫能とに対する免疫抑制が質的に異っていることを示している. さらに, 特異的免疫能は腫瘍増殖により抑制されるもののガラス附着細胞を除くことにより回復させることができることから, 担癌宿主の自己のリンパ球から抗腫瘍能を有するものを誘導し, 特異的免疫療法を行うことができる可能性を示唆している.