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Effect of serum fractions obtained from cancer patients by double filtration plasmapheresis combined with natural tumor necrosis factors and cyclophosphamide on murine pulmonary metastases.

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Abstract

We investigated the effects of fractionated sera obtained from cancer patients by double filtration plasmapheresis (DFPP) plus antitumor agents on murine pulmonary metastasis. Fractions of the sera, in combination with natural human tumor necrosis factors (nTNF) and cyclophosphamide (Cy), were systemically administered to Lewis lung carcinoma-bearing mice. When the second filtrate (a plasma fraction containing substances composed of smaller molecular weight compounds) combined with low-dose nTNF (1,000 U/kg) and Cy (250 micrograms/kg) was administered to the mice, the degree of metastasis was significantly suppressed compared with the control group (p less than 0.01). In contrast, the discarded fluid (a plasma fraction containing larger molecular weight compounds) combined with the same doses of nTNF and Cy caused little inhibition of metastasis. Also, the discarded fluid significantly suppressed natural killer activity compared with normal sera (p less than 0.01). The results suggested that DFPP combined with nTNF and Cy is an efficient procedure to remove immunosuppressive factors from the sera of cancer-bearing hosts, to enhance the host antitumor immunity, and to suppress tumor proliferation.

KEYWORDS: double filtration plasmapheresis, serum fractions, tumor necrosis factors, cyclophosphamide, synergistic effect

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Effect of Serum Fractions Obtained from Cancer Patients by Double Filtration Plasmapheresis Combined with Natural Tumor Necrosis Factors and Cyclophosphamide on Murine Pulmonary Metastases

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We investigated the effects of fractionated sera obtained from cancer patients by double filtration plasmapheresis (DFPP) plus antitumor agents on murine pulmonary metastasis. Fractions of the sera, in combination with natural human tumor necrosis factors (nTNF) and cyclophosphamide (Cy), were systemically administered to Lewis lung carcinoma-bearing mice. When the second filtrate (a plasma fraction containing substances composed of smaller molecular weight compounds) combined with low-dose nTNF (1,000 U/kg) and Cy (250 μg/kg) was administered to the mice, the degree of metastasis was significantly suppressed compared with the control group ($p<0.01$). In contrast, the discarded fluid (a plasma fraction containing larger molecular weight compounds) combined with the same doses of nTNF and Cy caused little inhibition of metastasis. Also, the discarded fluid significantly suppressed natural killer activity compared with normal sera ($p<0.01$). The results suggested that DFPP combined with nTNF and Cy is an efficient procedure to remove immunosuppressive factors from the sera of cancer-bearing hosts, to enhance the host antitumor immunity, and to suppress tumor proliferation.

Key words : **double filtration plasmapheresis, serum fractions, tumor necrosis factors, cyclophosphamide, synergistic effect**

Reports from our laboratory have documented that various immunosuppressive factors (IFs) in the sera of cancer-bearing hosts can be removed by double filtration plasmapheresis (DFPP) and that the suppressed immunity of the hosts against tumors is augmented after DFPP (1-4). Recently, we have demonstrated that the discarded fluid (a plasma fraction containing larger molecular weight compounds) which was removed by DFPP promoted the growth and the metastases of tumors in mice (5). In place of plasma exchange (6, 7), it is a new method which does not require a lot of fresh frozen plasma (FFP) as a substitution fluid. However, conventional DFPP therapy does not adequately suppress tumor growth. Therefore, it is thought that antitumor agents should be simultaneously used with DFPP to cause tumor regression. Though most anti-cancer agents suppress host immunity, it has been demonstrated that certain agents such as tumor necrosis factors (TNF) and cyclophosphamide (Cy) are able to augment host antitumor

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immunity under specific conditions (8, 9). In this study, we examined the effects of fractionated sera obtained by DFPP on murine pulmonary metastases in combination with natural human TNF (nTNF) and Cy. Also, the immunological effect of the fractionated sera on natural killer (NK) activity was investigated.

Materials and Methods

Drugs. nTNF was supplied by Hayashibara Biochemical Laboratories Inc., Okayama, Japan and injected intravenously into mice. The protein nTNF has a molecular weight of 17,000, is stable at pH between 5.2 and 6.2 at 52°C for 30 min, and consists of 161 amino acids (10). Its cytotoxicity was determined by the highly-sensitive and rapid assay of Eifel *et al.* (11) for lymphotoxin using mice L929 cells, and expressed as the reciprocal of the dilution that produced a cytopathic effect (CPE) in 50% of the target cells. Cy (Sigma Chemical Company, St. Louis, MO, USA) was dissolved in sterile saline and injected intraperitoneally into mice.

Mice. Female C57BL/6 mice 6 weeks of age were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan).

Tumor. Lewis lung carcinoma (3LL), which is syngeneic to C57BL/6 mice and induces lung metastases, was maintained *in vivo* by intramuscular injection into the femoral region of C57BL/6 mice.

Fractionation of cancer patients' serum by DFPP. The serum fractions used in this experiment were obtained from cancer patients who underwent DFPP at the First Department of Surgery of Okayama University Medical School (1). All patients had metastatic disease that was beyond surgical cure. Blood from the femoral vein was circulated extracorporally at 100 ml/min. The blood was separated with the first filter (Plasmacure, Kuraray Company, Osaka, Japan) into a cellular component and a plasma component (the first filtrate). The first filtrate (fraction 1, 1F) was filtered through the second filter (Evaflux 3A, Kuraray Company, Osaka, Japan), and separated into fraction 2 (the discarded fluid, DF) containing substances with a larger molecular weight and fraction 3 (the second filtrate, 2F) containing substances with a smaller molecular weight, including albumin. Fraction 3 together with the cellular component was infused back into the patient. Fraction 2 was discarded and replaced with substitution fluid such as 5% human albumin solution as described (5). Fraction 1 (1F), fraction 2 (DF) and fraction 3 (2F) were used in the following experiments.

Effect of cancer patients' serum fractionated by DFPP on NK activity. Peripheral blood lymphocytes (PBL) for use as effector cells were isolated by the Ficoll-Conray density gradient technique from the heparinized blood of healthy adults (12). The PBL, which were suspended in RPMI 1640 (Nissui Pharmaceutical Company, Tokyo, Japan)-HEPES (Sigma Chemical Company, St. Louis, MO, USA) containing 10% fetal calf serum, were preincubated with the fractionated sera at a final concentration of 50%. Target cells, the human hemopoietic cell line K562, were labeled by incubation with $Na_2{}^{51}CrO_4$ for 1 h. The ^{51}Cr-labeled target cells (T) were mixed with the effector cells (E) at a T : E cell ratio of 1 : 50 in 96-well microtiter plates, and then incubated for 4 h. Following incubation, the supernatant of each well was collected and the radioactivity released was determined with a gamma counter (Aloka Company, Tokyo, Japan). Percent inhibition was calculated as follows :

$$\%\ \text{inhibition} = \frac{\%\ \text{cytotoxicity of control} - \%\ \text{cytotoxicity of tested sera}}{\%\ \text{cytotoxicity of control}} \times 100,$$

where

$$\%\ \text{cytotoxicity} = \frac{\text{test cpm} - \text{spontaneous cpm}}{\text{total cpm} - \text{spontaneous cpm}} \times 100.$$

Sera from healthy donors were used as the control. Spontaneous ^{51}Cr-release from the K562 target cells was never greater than 10% under any of the conditions used.

Experimental design of mice pulmonary metastatic tumor. C57BL/6 mice were inoculated in the left foot pad with 1×10^6 3LL cells to produce a primary tumor. The primary tumor was removed by femoral amputation under ether anesthesia on the 10th day following inoculation. After the amputation, the mice were randomly assigned to groups. Serum fractions combined with nTNF or Cy were administered daily to mice via the tail vein from the day after the amputation for 10 days. Saline was used as the control fluid in this experiment. The pulmonary metastatic tumors were evaluated on the 21st day after inoculation. The evaluation was carried out by Wexler's method (13). In brief, the lungs were excised in one piece, dyed with India ink, washed for 5 min with flowing water, bleached and fixed in Fekete's solution for 24 h. The number of metastatic tumors was counted, and statistical analysis of significance of differences was

performed by Student's t-test.

Results

Effect of cancer patients' serum fractionated by DFPP on NK activity. The inhibition rate of the first filtrate was 37.3 ± 11.5 %, while that of the second filtrate was 12.8 ± 8.7 %, which was significantly lower than that of the first filtrate ($p<0.01$). In contrast, the discarded fluid inhibited NK activity of normal lymphocytes by 75.6 ± 14.7 %, which was significantly greater than that of the first filtrate ($p<0.01$). The % cytotoxicity of the control group was 26.6 ± 6.5 % (Fig. 1).

Effects of DFPP fractionated sera on pulmonary metastases of Lewis lung carcinoma in mice. The number of lung metastases was 42.8 ± 13.4 in the control group, while it was 68.7 ± 15.1 in the discarded fluid (D) group, significantly greater than in the control group ($p<0.01$). It was 36.0 ± 10.0 in the second filtrate group, but not significantly less than in the control group (Fig. 2).

Effects of low-dose Cy on metastases in cancer-bearing mice. The number of lung metastatic foci in mice administered 500 μg/kg, 1,000 μg/kg and 2,000 μg/kg of Cy was 15.4 ± 12.2, 9.7 ± 9.8 and 6.7 ± 8.7, respectively. At each dose, the number of metastases was significantly decreased compared with the control group ($p<0.01$). The results suggested that low-dose Cy dose-dependently suppressed tumor

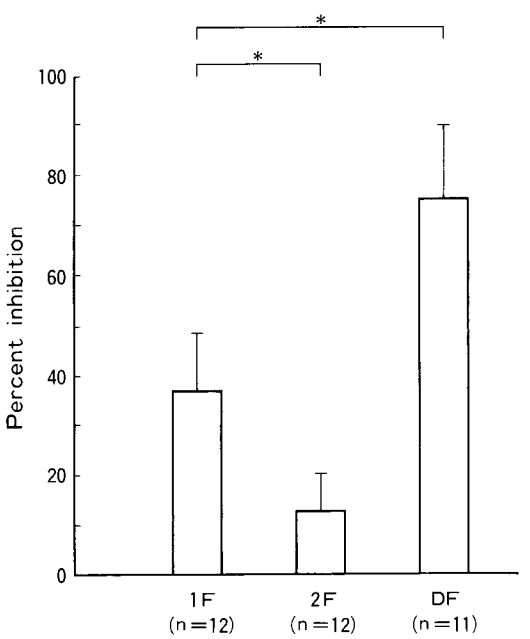

Fig. 1 Inhibitory effect of cancer patient serum fractionated by double filtration plasmapheresis (DFPP) on natural killer activity. The first filtrate (1F), the second filtrate (2F) and the discarded fluid (DF) were obtained by DFPP. Sera from healthy donors were used as the control. Spontaneous ^{51}Cr-release from the K562 target cells was never greater than 10 % under any condition. *, Significantly different by Student's t-test, $p<0.01$.

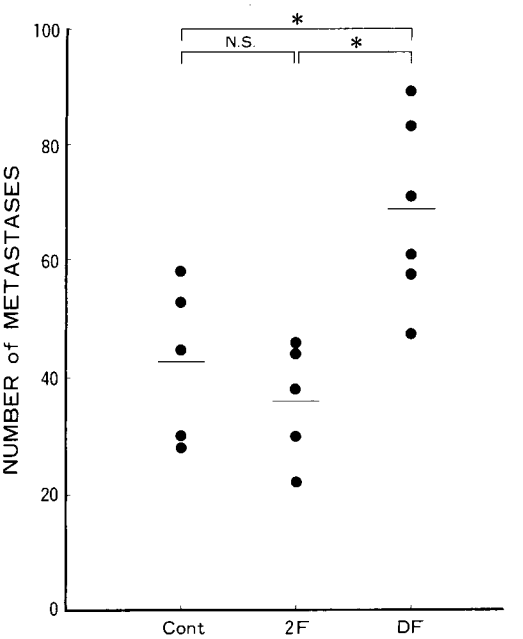

Fig. 2 Effects of the fractionated sera obtained from double filtration plasmapheresis on the pulmonary metastasis of Lewis lung carcinoma in mice. Each C57BL/6 mouse was injected with 1×10^6 Lewis lung carcinoma cells into a footpad. The limb with the tumor was amputated on the 10th day after the inoculation. The mice were randomly assigned to 3 groups. Saline (0.2 ml) (Cont ; n=5), the second filtrate (0.2 ml) (2F ; n=5) and the discarded fluid (0.2 ml) (DF ; n=6) were injected intravenously into mice via the tail vein daily from the day after the amputation for 10 days. The significance of differences in lung metastases among the 3 groups was determined by Student's t-test ; *, significantly different ($p<0.01$) ; N. S., not significantly different.

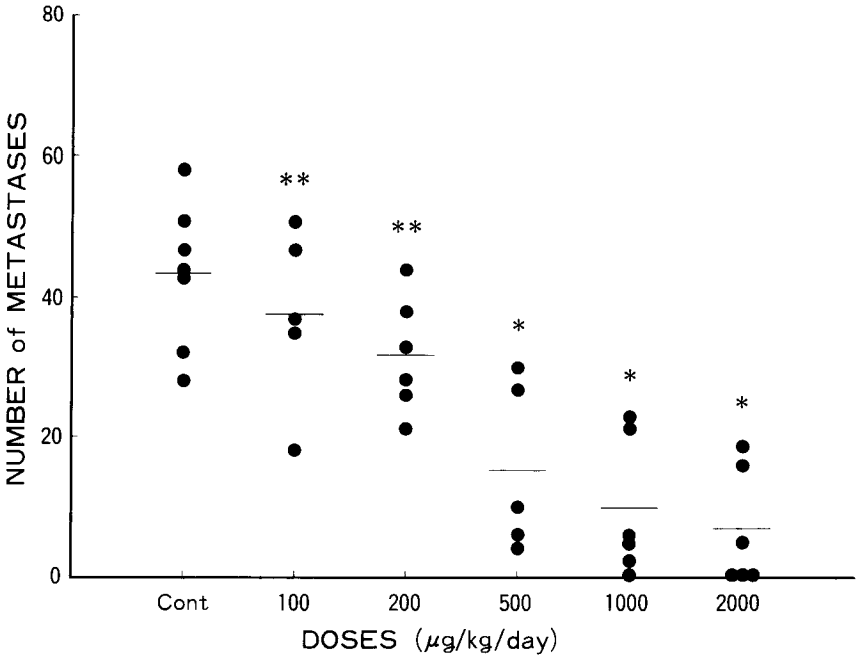

Fig. 3 Effects of low-dose cyclophosphamide on tumor metastasis in cancer-bearing mice. Each C57BL/6 mouse was injected with 1×10^6 Lewis lung carcinoma cells into a footpad. The limb with the tumor was amputated on the 10th day after the inoculation. The mice were randomly assigned to 6 groups. Saline (0.2 ml, Cont), cyclophosphamide at a dose of 100, 200, 500, 1,000 and 2,000 μg/kg of body weight were administered daily to mice intraperitoneally from the day after the amputation for 10 days. The significance of differences in lung metastases between the control and the cyclophosphamide-treated groups was determined by Student's *t*-test ; *, significantly different ($p<0.01$) ; **, not significantly different.

metastasis. Accordingly, we used a sub-effective dose of Cy (250 μg/kg) in the following experiments (Fig. 3).

Effects of serum fractions combined with nTNF on tumor metastases in cancer-bearing mice. The number of lung metastatic foci in mice administered 1,000 U/kg of nTNF was 26.3 ± 12.0, which was significantly lower than in the control group ($p<0.05$). The number of metastases in the discarded fluid+nTNF (500 U/kg) and the discarded fluid+nTNF (1,000 U/kg) groups was 63.0 ± 10.7 and 56.8 ± 10.9, respectively, and significantly increased compared with that in the control group. On the other hand, the number of metastases in the second filtrate+nTNF (500 U/kg) group was 30.5 ± 14.1, which was significantly lower than that in the discarded fluid+nTNF (500 U/kg) group ($p<0.01$). The number in the control group was 42.5 ± 7.2 (Fig. 4).

Effects of serum fractions combined with nTNF and Cy on tumor metastases in cancer-bearing mice. The number of lung metastases in the discarded fluid+Cy+nTNF group was 47.3 ± 7.9, which was similar to that in the control group. It was 10.1 ± 8.4 in the second filtrate+Cy+nTNF group, which was significantly decreased compared with the control group ($p<0.01$). The number in the second filtrate+Cy+nTNF group was significantly smaller than that in the group administered Cy alone ($p<0.05$) (Fig. 5).

Discussion

Natural killer cells are thought to play an important role in the immune defense system against tumors, especially in resistance to the dissemination of tumor cells (14, 15). Pross and

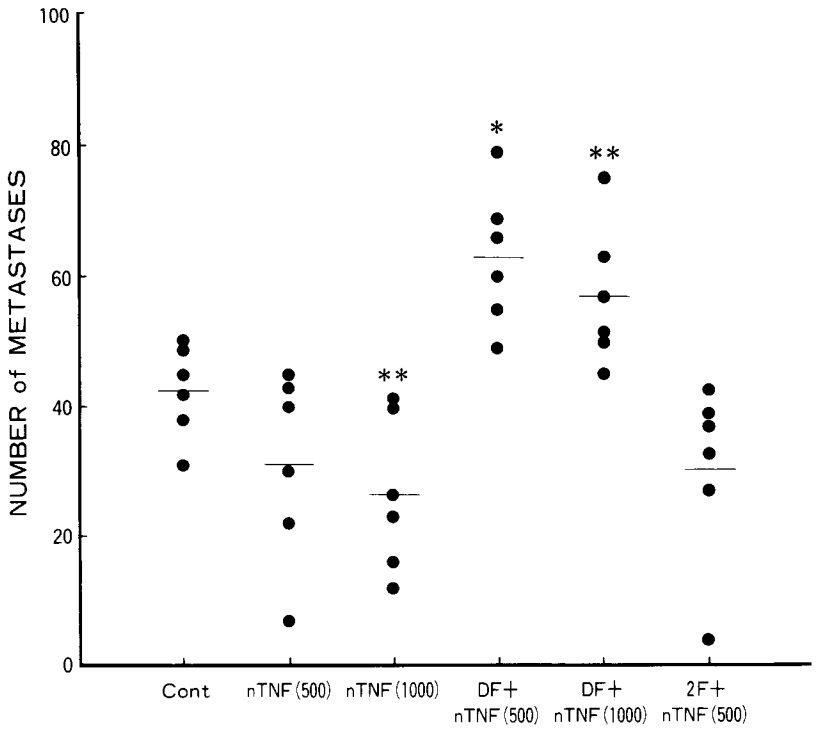

Fig. 4 Effects of serum fractions combined with natural human tumor necrosis factors (nTNF) on tumor metastasis in cancer-bearing mice. Each C57BL/6 mouse was injected with 1×10^6 Lewis lung carcinoma cells into a footpad. The limb with the tumor was amputated on the 10th day after the inoculation. The mice were randomly assigned to 6 groups. Saline (0.2 ml, Cont), nTNF (500 U/kg of body weight), nTNF (1,000 U/kg), the discarded fluid (DF, 0.2 ml)+nTNF (500 U/kg), DF (0.2 ml)+nTNF (1,000 U/kg) and the second filtrate (2F, 0.2 ml)+nTNF (500 U/kg) were injected intravenously into mice via the tail vein daily from the day after the amputation for 10 days. The significance of differences in lung metastases between the 6 groups was determined by Student's *t*-test ; * and * *, significantly different at a p value of 0.01 and 0.05, respectively.

Baines have previously reported that NK activity in cancer patients has a tendency to decrease, and that it decreases markedly when metastatic tumors spread widely (16). On the other hand, there are T lymphocyte defense systems against tumors, that is, cytotoxic T cells directly lyse tumor cells (17) and helper T cells secrete some lymphokines which mediate antitumor responses (18). These immunological defense systems seem to be inhibited by inhibitors in the sera of hosts with cancer. A number of specific and non-specific IFs existing in the sera of hosts with cancer have been reported (19–22). They are thought to increase in the sera of hosts with advanced cancer, diminishing the antitumor response of the host and promoting the tumor growth.

Plasma exchange and DFPP are methods for attempting to remove IFs from the serum of a cancer patient and to augment the immunological potency of the host. Though plasma exchange eliminates IFs from the serum of a cancer-bearing host, it requires a lot of FFP as the substitution fluid and so has not been widely employed. In contrast, DFPP is an efficient system which does not require much substitution fluid. However, DFPP is usually applied to patients with advanced cancer and cannot cause tumor regression adequately by itself. Accordingly, it needs to be combined with treatment with antitumor agents that can be expected to augment host immunity, such as nTNF and Cy.

In this study, the number of pulmonary metastases in the discarded fluid group of mice

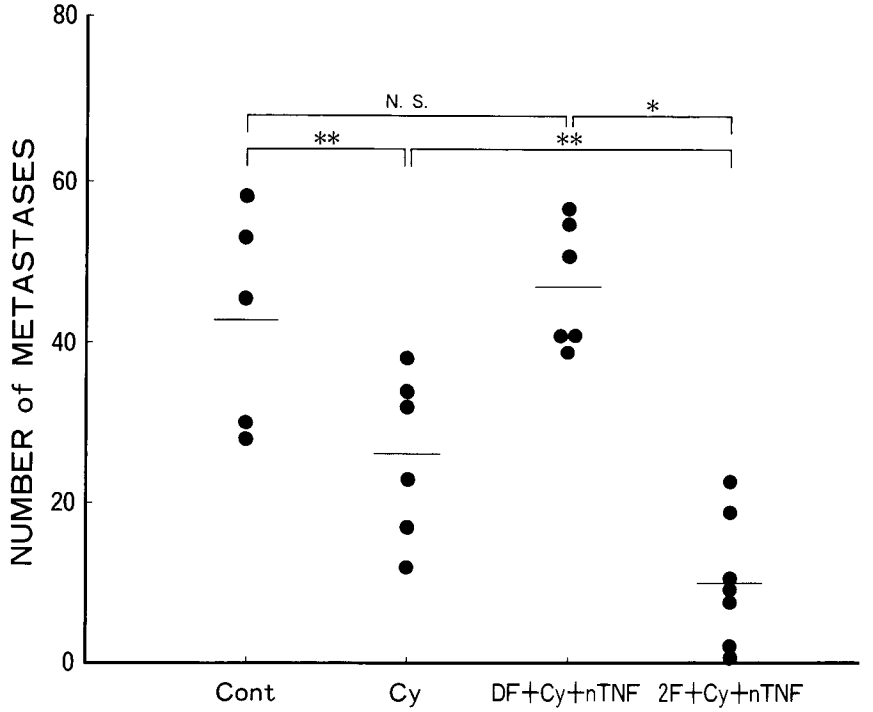

Fig. 5 Effects of serum fractions combined with natural tumor necrosis factors (nTNF) plus cyclophosphamide (Cy) on tumor metastasis in cancer-bearing mice. Each C57BL/6 mouse was injected with 1×10^6 Lewis lung carcinoma cells into a footpad. The limb with the tumor was amputated on the 10th day after the inoculation. The mice were randomly assigned to 6 groups. Saline (0.2 ml, Cont), Cy (250 μg/kg of body weight), the discarded fluid (DF, 0.2 ml)+nTNF (1,000 U/kg)+Cy (250 μg/kg) and the second filtrate (2F, 0.2 ml)+nTNF (1,000 U/kg)+Cy (250 μg/kg) were administered daily to mice from the day after the amputation for 10 days. The fractionated sera, saline and nTNF were injected intravenously. Cy was injected intraperitoneally. The significance of differences in lung metastases among the 4 groups was determined by Student's *t*-test ; * and * *, significantly different at a p value of 0.01 and 0.05, respectively ; N. S., not significantly different.

was shown to be significantly increased compared with that in the second filtrate group, which was similar to that in the control group. Previously we have investigated the effect of the serum fractions obtained from cancer(3LL)-bearing mice on murine pulmonary metastases. The fractions were the discarded fluid and the second filtrate, which were obtained from fractionation of pooled mouse sera with a mini second filter (pore size ; 0.02 μm). The administration of the discarded fluid to the mice resulted in the significant increase in the number of metastases ($p<0.01$), while the number of metastases in mice receiving the second filtrate was about the same as in mice receiving the control sera (23). The results suggested that tumor proliferating factors (one of the IFs) were present in significantly larger amounts in the discarded fluid group than in the second filtrate group and that the factors could be removed from serum by DFPP. Also, when serum fractions were administered in combination with low-dose nTNF and low-dose nTNF plus Cy, the number of lung metastases in the second filtrate+nTNF or the second filtrate+Cy+nTNF mice was drastically decreased compared with the discarded fluid+nTNF and the discarded fluid+Cy+nTNF mice. Furthermore, the number in the second filtrate+Cy+nTNF group was significantly decreased compared with that in the Cy only group. These results suggested that nTNF plus Cy had synergistic antitumor effects. On the contrary, the number of metastases in the

discarded fluid+Cy+nTNF group was never lower than in the control. Although nTNF (10) and Cy inhibited tumor metastasis dose-dependently, they were not so effective at low doses in diminishing lung metastases when the discarded fluid was administered to the host. Conversely, high doses of them are difficult to use because of severe side effects such as bone marrow suppression.

In the analysis of the inhibitory effect of the fractionated sera on normal lymphocytes, the NK activity of normal lymphocytes was shown to be strongly inhibited by the discarded fluid. In contrast, the second filtrate scarcely inhibited the activity at all. The data suggested that the discarded fluid contained a large amount of factors inhibitory to NK activity (NK inhibitory factor) in a significant amount, while the second filtrate did not. Previously we have demonstrated by HPLC analysis that the NK inhibitory factor with a molecular weight of 400,000 exists in the sera of cancer patients (23). It appears that DFPP can sufficiently remove the NK inhibitory factor from the serum and augment the NK activity of these patients. On the other hand, it has been reported that the factors inhibitory to PHA-induced blastogenesis (PHA inhibitory factors) are present in the serum fractions smaller than albumin and larger than albumin (2). Though the larger molecular weight fraction can be removed adequately by DFPP, the smaller molecular weight fraction is little affected by DFPP. It may be necessary to combine a specific adsorbent of the smaller molecular weight inhibitor with DFPP to remove the PHA inhibitory factors adequately. We are investigating the clinical applications of DFPP combined with chemotherapy and a glass-bead adsorbent which can adsorb the smaller molecular weight inhibitor.

In conclusion, it is suggested that DFPP combined with biological response modifiers such as nTNF and chemotherapeutic agents such as Cy, is an effective system for improving the immunological potency of cancer-bearing hosts and for inhibiting tumor metastatic proliferation. DFPP may enhance the efficacy of chemoimmunotherapy in cancer-bearing hosts.

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