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Changes in Lymphocyte Subsets Following Multiple Administration of Recombinant Interleukin-2 plus Recombinant Interferon-beta or -gamma in Tumor-bearing Mice

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Treatment with a combination of recombinant human interleukin-2 (rHIL-2) and recombinant mouse interferon-beta (rIFN-β) or -gamma (rIFN-γ) showed a significant antitumor effect against sc adenocarcinoma 755 in mice, although treatment with either one alone had almost no effect. The combination of rHIL-2 and rIFN-β caused regression of the tumor but the combination of rHIL-2 and rIFN-γ did not. Injection of tumor-bearing mice with the combinations of rHIL-2 and rIFN resulted in marked increases in the total number of peritoneal lymphocytes, and the frequency of Lyt-2$^+$ cells was more markedly increased by the combination of rHIL-2 and rIFN-β than by the combination of rHIL-2 and rIFN-γ. In Winn assay, elimination of the Lyt-2$^+$ population abolished the protective capacity of the peritoneal cells. The subsets of thymocytes were drastically changed when mice were bearing a tumor or were treated with cytokines. In particular, Lyt-2$^+$/L3T4$^+$ cells were decreased in tumor-bearing mice, but many Lyt-2$^+$/L3T4$^+$ cells were maintained in the thymus by treatment with a cytokine alone. When treated with rHIL-2 and rIFN-β, the Lyt-2$^+$/L3T4$^+$ cells were markedly decreased, while Lyt-2$^-$/L3T4$^-$ T-cells were increased, but these subsets were little changed by treatment with rHIL-2 plus rIFN-γ. Thus, injections of rHIL-2 and rIFN-β into tumor-bearing mice resulted in a high frequency of Lyt-2$^+$/L3T4$^-$ cells in the peritoneal cavity, together with changes in the T-cell subsets in the thymus. These results suggest that maturation of T-cells in the thymus may be an important step in the pathway by which cytokine treatment brings about regression of tumors.

Key words: Interleukin-2 — Interferon-β — Interferon-γ — Antitumor effect — Lyt-2$^+$ T subset

Interleukin-2 (IL-2),[4] which is a lymphokine predominantly produced by helper T cells, induces both proliferation and the cytotoxic functions of T lymphocytes and natural killer (NK) cells. A role of IL-2 in the growth of cytolytic T lymphocytes (CTL) is widely accepted, but the need for additional factors in the generation of a cytotoxic response *in vivo* is still controversial because injection of IL-2 alone does not induce proliferation of mature T lymphocytes but increases production of NK cells.[1)]

In our studies,[2,3)] recombinant human IL-2 (rHIL-2) treatment has shown weak antitumor activity against murine tumor adenocarcinoma 755 in C57BL/6 mice, whereas the combination of rHIL-2 and recombinant mouse interferon-beta (rIFN-β) has achieved complete eradication of the tumor after repeated administration. However, this combination did not cause regression of the tumor in athymic mice.[2)] On the other hand, the combination of rHIL-2 and recombinant mouse interferon-gamma (rIFN-γ) has shown marked retardation of the tumor but not regression.[3)] Regression of the tumor by the treatment with rHIL-2 plus rIFN-β may involve T-cell activation. Previously, various studies have emphasized that Lyt-1$^-$2$^+$ marker cells kill tumor cells, and some investigators have reported that Lyt-1$^+$2$^-$ [4-6)] or Lyt-1$^+$2$^+$ [7)] T-cells were also involved in *in vivo* tumor rejection. In this study, we employed anti-L3T4 antibody in addition to anti-Lyt-1 antibody. When stained with monoclonal antibodies directed against the T-cell differentiation antigens Lyt-2 and L3T4, at least four subpopulations of mouse thymocytes were able to be characterized: Lyt-2$^+$/L3T4$^-$ (CTL phenotype), Lyt-2$^-$/L3T4$^+$ (helper T-cell phenotype), Lyt-2$^+$/L3T4$^+$ and Lyt-2$^-$/ L3T4$^-$ (immature T-cell phenotype). We then decided to investigate the variation in surface markers of the lymphocytes following administration of rHIL-2 alone and with each of rIFN-β and rIFN-γ.

MATERIALS AND METHODS

Animals Inbred, 5-week-old, male C57BL/6 mice of approximately 22 g body weight were obtained from the Shizuoka Laboratory Animal Center (Hamamatsu). Each group consisted of 6 mice. These mice were maintained under specific-pathogen-free conditions in our laboratory. All experiments were initiated when the mice were 7 weeks old.

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[4] Abbreviations: IL-2, interleukin-2; rHIL-2, recombinant human interleukin-2; rIFN-β, recombinant mouse interferon-beta; rIFN-γ, recombinant mouse interferon-gamma; NK, natural killer; CTL, cytolytic T lymphocytes; mAb, monoclonal antibody(ies)

Tumor Adenocarcinoma 755 (5×10^5 cells/mouse) was implanted sc into the right hind legs of the mice, causing an sc tumor nodule to appear at the inoculation site in all animals on day 5.

Cytokines and treatment Lyophilized rHIL-2 (specific activity: 1×10^7 U/mg protein) was kindly supplied by Biogen S.A. Company, Switzerland, and Shionogi & Co., Osaka. rIFN-β (specific activity: 5.5×10^7 IU/mg protein) and rIFN-γ (specific activity: 4.6×10^6 U/mg protein) were generously provided by Toray Industries Inc., Kamakura.

When the tumor became palpable (about 5 mm in diameter), rHIL-2 and rIFN-β or -γ were administered ip in doses of 10^5 U/mouse and 10^5 IU/mouse, respectively, and this was continued daily for 9 days.

Monoclonal antibodies (mAb) Four different rat mAb specific for T-cell surface antigens were used: IgG_{2b} anti-Thy1.2 (mAb 30-H 12), IgG_{2a} anti-Lyt-1 (mAb 53.7.3), IgG_{2a} anti-L3T4 (mAb GK 1.5) and IgG_{2a} anti-Lyt-2 (mAb 53.6.7) mAb were purchased from Becton Dickinson (Mountain View, CA). In addition, IgG_{2b} murine/human anti-Mac-1 (M1/70), IgG_{2b} anti-Ia (M5/114) and anti-asialo GM_1 mAb were purchased from Hybritech Inc. (San Diego, CA) and Wako Pure Chemical Industries (Osaka), respectively. For flow cytometric analysis, anti-Mac-1, anti-Ia and anti-asialo GM_1 mAb were used together with fluorescein isothiocyanate (FITC)-coupled anti-rat and anti-rabbit Ig antibodies.

Lymphocyte subset analysis Organ studies were performed 6 h after the 9th daily ip injection of cytokines. The mice were killed by cervical dislocation, 10 ml of 0.9% saline solution was injected ip, and after gentle lavage the peritoneal contents were taken. The peritoneal contents obtained from three mice were pooled, washed to remove cellular debris, suspended in 5 ml of Cytotoxicity Medium containing 0.3% fetal calf serum (Cedarlane Laboratories Ltd., Hornby, Ontario), and stored on ice until determination of the subsets. The tumor, spleen, thymus and liver were rapidly removed, weighed and stored on ice (6 mice/group). The spleen and thymus were minced with scissors in separate Petri dishes containing 5 ml of Cytotoxicity Medium and pressed through a wire mesh (120 mesh), and the cells obtained were suspended in 15 ml of the medium on ice. For single-color analysis, fluorescein-labeled mAb was added to the cell suspension in a tube (about 1×10^5 cells/0.1 ml) at 4°C for 30 min and FACS lysing solution (Becton Dickinson) was added for 10 min. After centrifugation, saline was added (1 ml) and 5,000–20,000 cells from each suspension were analyzed by flow cytometry (Spectrum III, Ortho Diagnostic System Inc., MA). For two-color analysis, cells were incubated with fluorescein phycoerythrin (PE)-conjugated anti-L3T4 mAb plus fluorescein FITC-conjugated anti-Lyt-2 mAb. The proportions of Lyt-2^+/L3T4^-, Lyt-2^-/L3T4^+, and Lyt-2^+/L3T4^+ T-cells were determined directly as percentages. The proportion of Lyt-2^-/L3T4^- T-cells was calculated as the difference between the percentage of Thy-1.2^+ cells and the sum of the percentages of L3T4^+ and Lyt-2^+ minus Lyt-2^+/L3T4^+ cells.[8] The number of specific lymphocytes was calculated from the frequency of the specific lymphocytes and the total number of lymphocytes in the peritoneal cavity.

Winn assay Peritoneal cells were harvested 6 h after the last treatment with rHIL-2 plus rIFN-β (9 injections). Collected unfractionated peritoneal cells were washed three times in Cytotoxicity Medium. To obtain nonadherent peritoneal cells the unfractionated peritoneal cells were incubated in a gelatin-coated dish (Iwaki Glass, Tokyo) for 1 h at 37°C. The cells remaining in the supernatant after the second incubation were designated "nonadherent peritoneal cells." For pretreatment of peritoneal cells with mAb and Low-Tox-M rabbit complement (Cedarlane Laboratories Ltd.) the peritoneal cells were suspended in diluted mAb (2.5×10^6/ml) and placed at 25°C for 45 min. The cells were washed twice then suspended in complement (C)-containing medium (dilution, 1/10) and incubated for 45 min at 37°C. Subsequently the peritoneal cells were washed twice. Adenocarcinoma 755 tumor cells were adjusted to 5×10^5 viable single cells/tube. Winn assay was done as reported by Shimizu and Shen.[7]

Data analysis The reported results are the averages of experiments performed at least in duplicate under identical conditions. Student's t test was used to determine the statistical significance of differences.

RESULTS

Antitumor effects of rHIL-2 and/or rIFN-β or -γ on sc adenocarcinoma 755 Adenocarcinoma 755-bearing mice were administered rHIL-2 and/or rIFN-β or -γ by daily injection beginning on day 7 and continuing through day 15. The mice administered cytokines alone did not show any marked reduction in the tumor size (measured in terms of the tumor weight on day 15). When rHIL-2 and rIFN were administered together, tumor reduction was more significant than with either cytokine alone. Especially, combined treatment of mice with rHIL-2 and rIFN-β resulted in the greatest reduction in tumor size (T/C=34% vs rHIL-2 (T/C=79%) or rIFN-γ (T/C=90%), $P<0.001$). The combination of rHIL-2 and rIFN-γ also enhanced the antitumor effect (T/C=50% vs rHIL-2 or rIFN-γ (T/C=76%), $P<0.001$), but the potency of synergism was weaker than with the combination of rHIL-2 and rIFN-β.

Effect of rHIL-2 and/or rIFN-β or -γ on the thymus, spleen and liver The thymic and splenic reactions follow-

ing injections of cytokines in tumor-bearing mice were studied. The thymus decreased markedly in size following implantation of the tumor (52 ± 2 mg (SE) vs 14 ± 1 mg, $P<0.001$). No significant differences in the thymus weight were found between rIFN-γ-treated mice and untreated tumor-bearing mice, but the thymic enlargement was marked in mice administered rHIL-2 (24 ± 2 mg, $P<0.001$). Moreover, the thymus weight was further increased by the combination of rHIL-2 and rIFN-β compared to rHIL-2 alone (32 ± 2 mg, $P<0.05$), but not by the combination of rHIL-2 and rIFN-γ (22 ± 2 mg). On the other hand, the spleen of tumor-bearing mice was markedly increased in size compared to normal mice (about 5-fold). Treatment with either cytokine alone caused no significant change in the spleen size, but after the combination of rHIL-2 and rIFN-β the spleen was significantly smaller than after rHIL-2 alone (280 ± 15 mg vs 391 ± 18 mg, $P<0.001$); the combination of rHIL-2 and rIFN-γ did not cause this reduction in spleen size. Furthermore, the liver weight after treatment with the cytokines was affected similarly to the spleen weight. Thus, the sizes of these lymphoid organs, i.e., the thymus, spleen and liver, were drastically affected by these treatments with cytokines.

Changes in lymphocyte subsets in peritoneal lymphocytes following treatment with rHIL-2 and rIFN-β or -γ

In vivo studies[2, 3] suggest that one of the effector cells causing regression of tumors is CTL. We studied the changes in the lymphocyte subsets in lymphoid organs following administration of these cytokines. Lymphocytes possess Lyt-antigenic phenotypes which are characteristic of helper T-cells (Lyt-2^-/L3T4$^+$) and CTL (Lyt-2^+/L3T4$^-$). Lyt-antigenic phenotypes might therefore be of use in characterizing the effector cells for the combination of rHIL-2 and rIFN-β or -γ.

First, we studied whether the lymphocytes in the peritoneal cavity were increased and their phenotypes were changed following treatment with cytokines. Numerous lymphocytes were seen in the peritoneal cavity and the number was markedly increased by the combination of rHIL-2 and rIFN, especially by the combination of rHIL-2 and rIFN-γ (Table I).

Flow cytometric analysis of responding peritoneal-cavity lymphocytes was carried out to look for the presence of specific lymphocyte subpopulations defined by monoclonal antibodies. The frequencies of Lyt-1 and Lyt-2 marker cells in tumor-bearing mice were higher than in normal mice, but the frequencies of Mac-1, asialo GM_1 and Ia marker cells in tumor-bearing mice were less than in normal mice (Fig. 1).

The lymphocyte subsets following treatment with cytokines showed different patterns. When rHIL-2 was administered to tumor-bearing mice, the frequencies of Thy-1.2, L3T4, Lyt-2, Mac-1, asialo GM_1 and Ia marker cells were increased. When rIFN-β or -γ was administered to tumor-bearing mice, the frequencies of Thy-1.2^+, Mac-1^+ and asialo GM_1^+ cells were markedly increased. The frequency of Thy-1.2^+ cells after treatment with rIFN-γ (50%) was higher than with rIFN-β (28%). The combination of rHIL-2 and rIFN-β resulted in increased frequencies of many subsets of lymphocytes; in particular, the frequency of Lyt-2^+ cells was significantly increased compared to treatment with rHIL-2 alone ($P<0.01$), and after the combination of rHIL-2 and rIFN-γ there was only a small increase. Among the various cytokine treatments, rHIL-2 plus rIFN-γ caused the largest increase in cell number of each subset (Table II).

Table I. Number of Lymphocytes in Peritoneal Cavity Following Treatments with rHIL-2, rIFN-β and rIFN-γ

Treatment[a]	Number of lymphocytes[b] ($\times10^3$ cells/mouse)	T/C[c]
Normal mice	120 ± 34	—
Tumor-bearing mice (Control)	500 ± 230	1.0
rHIL-2, 10^5 U/mouse	780 ± 130	1.6
rIFN-β, 10^5 IU/mouse	670 ± 120	1.3
rIFN-γ, 10^5 U/mouse	910 ± 200	1.8
rHIL-2+rIFN-β	2160 ± 580	4.3
rHIL-2+rIFN-γ	3560 ± 950	7.1

a) rHIL-2 and/or rIFN-β or -γ were administered to tumor-bearing mice on days 7–15. The mice were killed 6 h after the last treatment and peritoneal cells were taken.
b) Mean $\pm$ SE of 4 experiments (three mice/group).
c) Determined as the ratio of mean lymphocyte number of the treated group to that of the untreated one (control).

Effects of rHIL-2 and rIFN-β or -γ on lymphocyte subsets in the thymus and spleen The thymus and spleen sizes were drastically changed following treatment with cytokines. The thymus is regarded as the differentiation site for T-cell lymphopoiesis. In two-color staining of the total thymocytes with antibodies specific for Lyt-2 and L3T4, four populations were clearly discernible by the use of anti-Lyt-2 and anti-L3T4 antibodies: Lyt-2^+/L3T4$^-$, Lyt-2^-/L3T4$^+$, Lyt-2^+/L3T4$^+$ and Lyt-2^-/L3T4$^-$. Almost all thymocytes in normal mice were Thy-1.2^+ cells (99%) and Lyt-2^+/L3T4$^+$ cells amounted to 90%: most of such "double-positive" cells are destined to die *in situ*.[9] A small subpopulation comprising 2–3% of the total lymphocytes expressed neither Lyt-2 nor L3T4 (Lyt-2^-/L3T4$^-$), and they have been proposed as putative T-cell precursors.[10, 11] The two mature phenotype subpopulations, Lyt-2^-/L3T4$^+$ and Lyt-2^+/L3T4$^-$ cells, represented 6% and 2% of the total thymocyte population, respectively (Fig. 2).

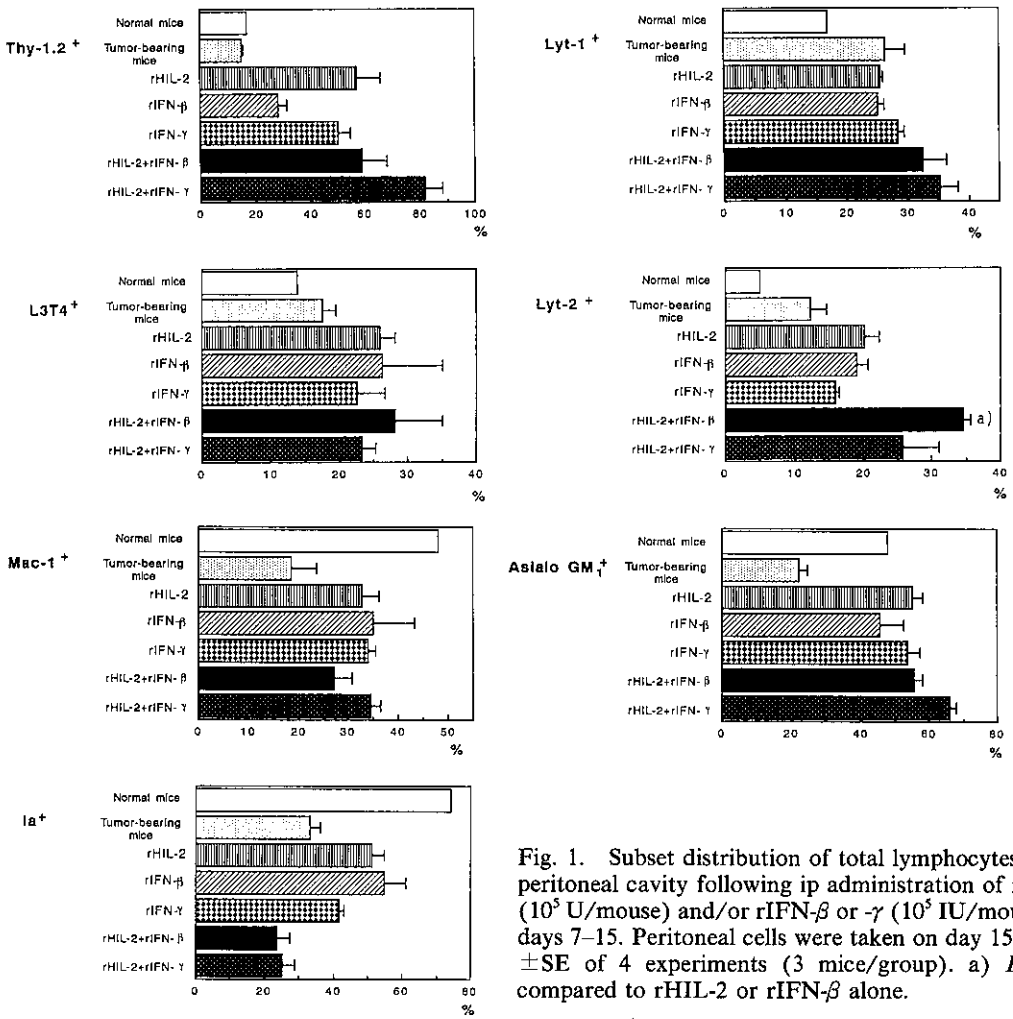

Fig. 1. Subset distribution of total lymphocytes in the peritoneal cavity following ip administration of rHIL-2 (10^5 U/mouse) and/or rIFN-β or -γ (10^5 IU/mouse) on days 7–15. Peritoneal cells were taken on day 15. Mean $\pm$SE of 4 experiments (3 mice/group). a) $P<0.01$ compared to rHIL-2 or rIFN-β alone.

Table II. Flow Cytometric Analysis of Lymphocytes in Peritoneal Cavity of Tumor-bearing Mice after Treatments with rHIL-2 and rIFN-β or -γ

Treatment[a]	Positive cell number ($\times 10^3$ cells/mouse)[b]						
	Thy-1.2$^+$	Lyt-1$^+$	L3T4$^+$	Lyt-2$^+$	Mac-1$^+$	Asialo GM$_1^+$	Ia$^+$
Normal mice	24±1	24±4	19±2	7±2	67±3	62±20	105±17
Tumor-bearing mice	92±19	168±41	100±16	81±2	99±19	142±33	238±29
rHIL-2	420±40	238±30	206±45	150±16	260±58	430±67	482±124
rIFN-β	190±45	183±46	184±70	100±29	244±76	318±78	420±2
rIFN-γ	435±67	257±59	230±83	145±34	304±60	465±77	521±7
rHIL-2+rIFN-β	1358±519	661±161[c]	656±245[d]	619±190[c]	629±219[d]	1239±393[d]	709±7
rHIL-2+rIFN-γ	3015±1002[e]	1294±406[e]	783±136[e]	939±315[e]	1243±349[e]	2307±593[e]	633±162

a) rHIL-2 (10^5 U/mouse) and rIFN-β (10^5 IU/mouse) or rIFN-γ (10^5 U/mouse) were administered to tumor-bearing mice on days 7–15. The mice were killed 6 h after the last treatment and the peritoneal cells were taken.
b) Mean$\pm$SE of four experiments (three mice/group).
c) $P<0.05$ compared to rHIL-2 or rIFN-β alone.
d) $P<0.01$ compared to rHIL-2 or rIFN-β alone.
e) $P<0.05$ compared to rHIL-2 or rIFN-γ alone.

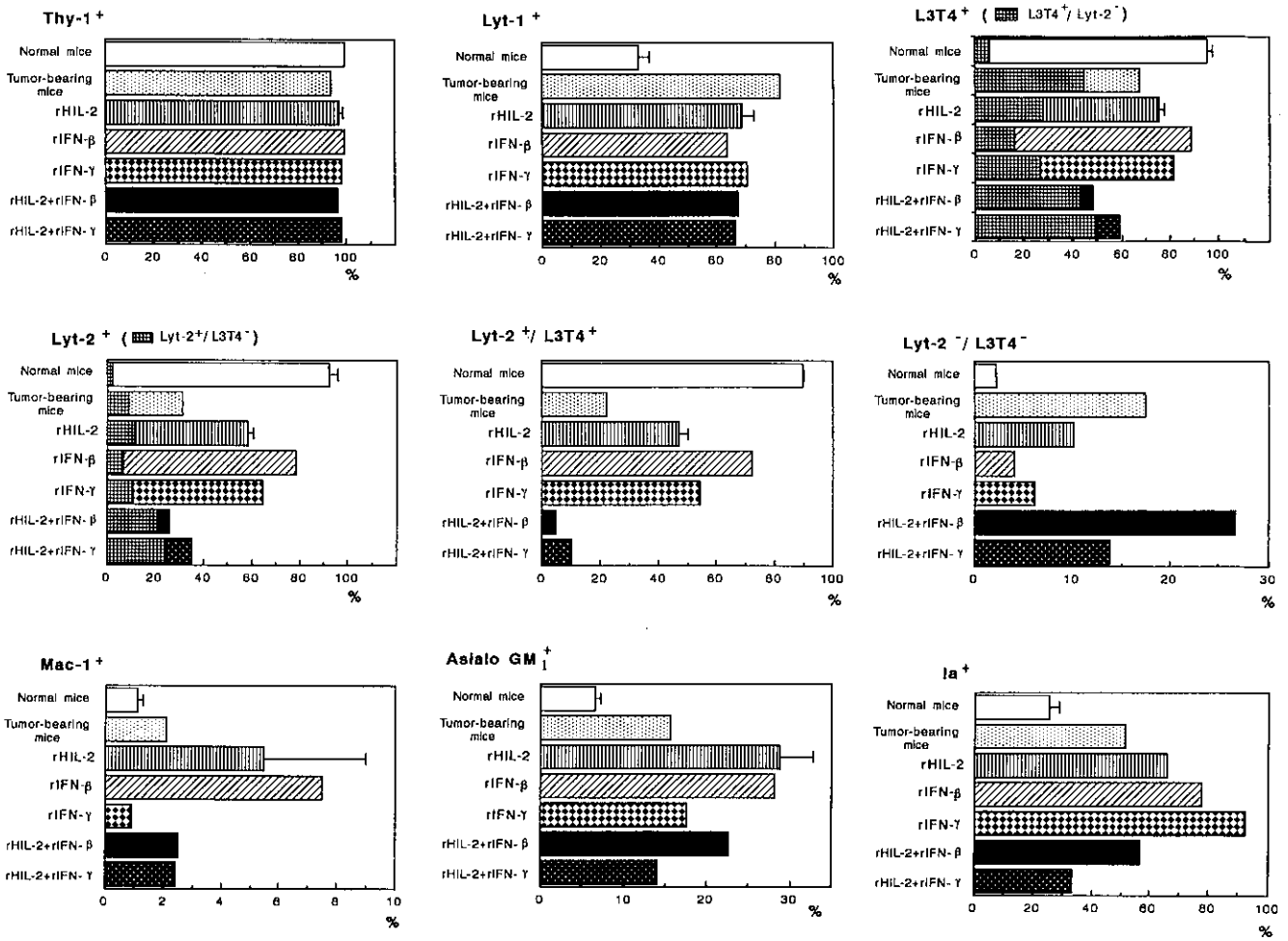

Fig. 2. Subset distribution of total thymocytes following ip administration of rHIL-2 (10^5 U/mouse) and/or rIFN-β or -γ (10^5 IU/mouse) on days 7–15. The thymus was taken on day 15. Mean±SE of 4 or 2 experiments (6 mice/group).

Tumor-bearing mice showed thymic involution. Frequencies of Lyt-1, asialo GM_1 and Ia marker cells in tumor-bearing mice were increased. Lyt-2^-/L3T4^+ and Lyt-2^+/L3T4^- cells were also increased compared to the populations in normal mice. Immature type Lyt-2^+/L3T4^+ marker cells were drastically decreased, while Lyt-2^-/L3T4^- T-cells were increased. rHIL-2, rIFN-β or -γ treatment inhibited the decrease in Lyt-2^+/L3T4^+ cells. The frequencies of Lyt-2^-/L3T4^+ and Lyt-2^-/L3T4^- T-cells were decreased. Other subsets showed no marked changes. In the combination of rHIL-2 and rIFN-β or -γ, which showed marked antitumor effects, the frequency of Lyt-2^+/L3T4^+ cells (5 and 10%, respectively) was less than that in the tumor-bearing mice (22%). Moreover, Lyt-2^-/L3T4^- and Lyt-2^+/L3T4^- T-cells were markedly increased by the combination of rHIL-2 and rIFN-β. However, the frequency of Lyt-2^-/L3T4^+ T-cells was unchanged following these combinations.

On the other hand, all the T-cell surface markers of splenocytes in tumor-bearing mice were decreased compared to those in normal mice while asialo GM_1 marker cells were increased. However, there were no marked differences in surface markers between treatment with a cytokine alone and in combination.

Winn assay An sc implanted (5×10^5 cells) adenocarcinoma 755 grew progressively in syngeneic C57BL/6 mice. Admixture of peritoneal cells from normal mice treated with rHIL-2 plus rIFN-β in ratios as high as 2:1 (peritoneal cells: tumor cells) did not protect the mice, but peritoneal cells from tumor-bearing mice treated with rHIL-2 plus rIFN-β completely protected them in ratios

Table III. Growth of Adenocarcinoma 755 Cells, Combined with Peritoneal Cells Treated with rHIL-2 plus rIFN-β in Normal and Tumor-bearing C57BL/6 Mice

Ratio of peritoneal cells to tumor cells	Source of peritoneal cells[a]		
	Tumor-bearing mice		Normal mice
	Total PC	NAPC[b]	Total PC
2:1[c]	0/4[d]	—	4/4
1:1	0/4	0/4	4/4
1/2:1	0/4	0/4	—
1/3:1	—	1/4	—
1/5:1	3/4	4/4	—
1/10:1	3/4	3/4	—

a) rHIL-2 (10^5 U/mouse) and rIFN-β (10^5 IU/mouse) were administered to tumor (adenocarcinoma 755)-bearing mice or normal C57BL/6 mice on days 7–15. The mice were killed 6 h after the last treatment and the peritoneal cells (PC) were taken.
b) NAPC=nonadherent peritoneal cells.
c) 5×10^5 adenocarcinoma 755 cells.
d) Proportion of mice with progressive tumor growth.

Table IV. Growth of Adenocarcinoma 755 Cells Combined with Total Peritoneal Cells Treated with Anti-Thy-1, Anti-Lyt or Anti-asialo GM_1 Antibody and Complement

Total peritoneal cells[a] treated with	Proportion of mice with progressive tumor growth
Medium	0/8
Medium+Complement (C)	0/7
Anti-Thy-1.2 mAb+C	8/8
Anti-Lyt-1.2 mAb+C	3/4
Anti-Lyt-2.2 mAb+C	7/8
Anti-L3T4 mAb+C	1/8
Anti-asialo GM_1 mAb+C	7/8

a) rHIL-2 (10^5 U/mouse) and rIFN-β (10^5 IU/mouse) were administered to tumor (adenocarcinoma 755)-bearing mice on days 7–15. The mice were killed 6 h after the last treatment and the peritoneal cells were taken. The numbers of peritoneal cells and tomor cells were 5×10^5 and 5×10^5, respectively.

as high as 1:2. Moreover, nonadherent peritoneal cells completely protected the mice (Table III). On the other hand, admixture of thymocytes or splenocytes from tumor-bearing mice treated with any cytokine (effector cells: tumor cells=10:1) gave no protection against adenocarcinoma 755.

Table IV shows the results of adding unfractionated peritoneal cells (5×10^5) that had been preselected by reaction with Thy-1.2, Lyt-1, Lyt-2, L3T4 or asialo GM_1 mAb and C before combining them with the tumor (5×10^5). Elimination of the Thy-1^+, Lyt-1^+, Lyt-2^+ and asialo GM_1^+ populations abolished the protection.

DISCUSSION

The combination of rHIL-2 and rIFN-β markedly enhances the individual antitumor effects of these cytokines, and repeated treatment achieves some cures in mice, whereas the combination of rHIL-2 and rIFN-γ does not.[3] The high incidence of complete regression of tumors may result from augmented induction of antitumor-specific immunity, including T-cells. The numbers of lymphocytes detectable in the peritoneal cavity following treatment with rHIL-2 plus rIFN-β or -γ were 4- or 7-fold higher than in untreated tumor-bearing mice, respectively. Combined administration of rHIL-2 and rIFN-γ was more effective in the induction of lymphocytes in the peritoneal cavity than combined administration of rHIL-2 and rIFN-β.

rHIL-2 is known to enhance both the growth and cytotoxic functions of T-lymphocytes and NK cells *in vitro*,[12] but Piguet *et al.*[1] reported that rHIL-2 treatment *in vivo* did not induce proliferation of mature T-lymphocytes. This suggests that maturation of T-lymphocytes *in vivo* requires other factors. T-cells are able to be classified on the basis of their Lyt surface markers. In lymphocytes in the peritoneal cavity, the frequency of Lyt-2 marker cells was markedly increased by the combination of rHIL-2 and rIFN-β. In Winn assay, progressive tumor growth was completely prevented by peritoneal cells from tumor-bearing mice treated with rHIL-2 plus rIFN-β, but not by peritoneal cells from normal mice. Elimination of Thy-1^+, Lyt-1^+, Lyt-2^+ or asialo GM_1^+ cells by mAb and complement abolished the protective capacity of the immune peritoneal cells, showing that immunity under these conditions is mediated by T-cells. Recently, Rosenberg *et al.*[13] reported that the combination of rHIL-2 and rIFN-αA/D showed a synergistic antitumor effect and this synergy was dependent on Lyt-2^+ T-cells. There is a possibility that the effector cells may be large mononuclear leukocytes with Thy-1.2^+, Lyt-2^+, L3T4$^-$ and asialo GM_1^+ as reported by Piguet *et al.*[1]

rIFN-γ has been found to prime macrophages for nonspecific tumoricidal activity[14–16] and to induce or enhance intracellular cytocidal reactions.[17] Nevertheless, in the regression of sc adenocarcinoma 755, the combination of rHIL-2 and rIFN-β was superior to the combination of rHIL-2 and rIFN-γ.[3] However, if rHIL-2 and rIFN-γ had been injected ip against an ip-implanted tumor, greater efficacy might have been shown, as reported by Silagi *et al.*,[18] because this combination induced a larger number of lymphocytes in the peritoneal cavity compared with the combination of rHIL-2 and rIFN-β. In an MCA sarcoma cell system, Agah *et al.*[19] reported that the synergistic effect of rIFN-γ with rHIL-

2 was dependent on the route of administration of rIFN-γ: synergism was shown in the ip route, but not in the iv route. rIFN-β and rIFN-γ may exhibit markedly different pharmacodynamics.

The thymus of adenocarcinoma 755-bearing mice became undersized, while the spleen was enlarged. When rHIL-2 was administered to tumor-bearing mice, the weights of the thymus, spleen and liver became significantly higher than in untreated tumor-bearing mice. When rHIL-2 and rIFN-β were administered together to tumor-bearing mice, the thymus became larger and the spleen smaller than when rHIL-2 was administered alone, but the combination of rHIL-2 and rIFN-γ did not change the weights of these organs. Thus, combined administration of rIFN-β and rHIL-2 had a greater effect on the lymphoid organs than combined administration of rIFN-γ and rHIL-2.

The thymus is the site of maturation of T-lymphocytes. The subsets of thymocytes in tumor-bearing mice were markedly different from those in normal mice. Frequencies of Lyt-2^-/L3T4$^+$, Lyt-2^+/L3T4$^-$ and Lyt-2^-/L3T4$^-$ T-cells were increased, but Lyt-2^+/L3T4$^+$ cells, which are a major subpopulation of the thymocytes in normal mice and are known to consist mostly of non-cycling cells, were decreased. However, Lyt-2^+/L3T4$^+$ immature T-cells in the thymus were maintained at a high percentage by treatment with rHIL-2, rIFN-β or -γ alone, although the percentage of Lyt-2^-/L3T4$^-$ T-cells was unchanged. These results may mean that one cytokine alone can hardly induce maturation of T-cells *in vivo*. Only when rHIL-2 and rIFN-β were combined, did Lyt-2^+/L3T4$^+$ cells decrease and Lyt-2^-/L3T4$^-$ T-cells increase markedly. The ip administration of rHIL-2 plus rIFN-β generated a much higher frequency of Lyt-2^+ cells in the peritoneal cavity than the combination of rHIL-2 and rIFN-γ. These findings suggest that both rHIL-2 and rIFN-β may play an important role in maturation of Lyt-2^+ cells by promoting self-renewal of stem cells within the thymus. Moreover, it seems possible that newly formed Lyt-2^+ cells arising from Lyt-2^-/L3T4$^-$ T-cells in the thymus response to the combination of rHIL-2 and rIFN-β migrate to the peritoneal cavity and also to tumors.

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