Cancer Immunol Immunother (1993) 36: 323–330

Cancer Immunology Immunotherapy

Prevention of lymph node metastases by adoptive transfer of CD4+ T lymphocytes admixed with irradiated tumor cells

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Received: 19 December 1991/Accepted: 1 December 1992

Abstract. CD4+8− T lymphocytes with potent antitumor activity in vivo were obtained in peritoneal exudate cells by immunizing mice with irradiated MM48 tumor cells admixed with OK-432. These immune CD4+ T cells were used in adoptive immunotherapy for prevention of lymph node metastases after removal of the primary tumor. Complete cure of metastases was obtained by adoptive transfer of CD4+ T cells admixed with irradiated MM48 tumor cells, but not by CD4+ T cells alone. To analyze the curative effect of admixing tumor cells on the prevention of metastases, a model of 1-day tumor inoculated with macrophages was used. Administration of immune CD4+ T cells alone resulted in the regression of local tumor in more than half of the mice, although all of them eventually died of lymph node metastases. On the other hand, adoptive transfer of immune CD4+ T cells plus irradiated tumor cells resulted in the complete regression of local tumors in all the mice, which survived without any sign of metastasis. The curative effect of the immune CD4+ T cells obtained by admixing irradiated tumor cells was tumor-specific. Macrophages induced by OK-432 (tumoricidal), implanted together with tumor, assisted tumor regression more than did macrophages elicited by proteose peptone (non-tumoricidal) in the same adoptive transfer system. Administration of recombinant interleukin-2 instead of stimulant tumor cells did not enhance, but rather eliminated the constitutive antitumor activity of CD4+ T cells. On the other hand, exogenous recombinant interleukin-1 was more effective in the enhancement of antitumor activity of the CD4+ T cells as compared with stimulant tumor cell administration. In this case, the activating states of macrophages at the implanted tumor site had no influence on the therapeutic efficacy. A possible role of macrophages for induction of tumor-specific cytotoxic T cells that were mediated by tumor-specific CD4+ T cells is discussed.

Key words: $CD4^+$ T cells – Adoptive immunotherapy – Interleukin-1 – Lymph node metastasis

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Introduction

It is widely accepted that CD4+ T lymphocytes show antitumor activity in vivo, though they have no cytotoxic activity [6, 7, 9]. Tumor-specific T cells react with tumor antigens and secrete lymphokine(s) leading to activation of macrophages as the final effector cells [19]. The activated macrophages kill tumor cells in a non-specific fashion in contrast with specific cytotoxic T lymphocytes [7].

Mouse ascites mammary tumor, MM48, cells are highly metastatic to the lymph nodes and are resistant to lymphokine-activated killer (LAK) and natural killer (NK) cells. Establishment of lymph node metastasis has been closely associated with the induction of tumor-specific suppressor T cells in the lymph node [25, 26].

Administration of low-dose cyclophosphamide to MM48-tumor-bearing mice leads to the inhibition of suppressor activity and a low incidence of lymph node metastasis [27]. Surgical resection of local tumor combined with cyclophosphamide administration resulted in a tumor-free survival rate of 50% in the treated mice.

In this report, we attempted to achieve the complete cure of all the tumor bearers without metastases by applying adoptive immunotherapy in combination with cyclophosphamide and surgical treatments.

Noncytolytic immune CD4+ T cells in peritoneal exudate cells against MM48 tumor cells showed a potent neutralizing activity in Winn's test. We used these CD4+ T cells for adoptive immunotherapy. Eradication of residual tumor cells was not achieved by the adoptive transfer of immune CD4+ T cells alone, but was completed when the CD4+ T cells were transferred with irradiated relevant tumor cells. It is speculated that antigenic stimulation and participation of some cytokines are necessary for extensive expression of the antitumor response of CD4+ T cells. The mechanism of this effective adoptive immunity is analyzed and discussed.

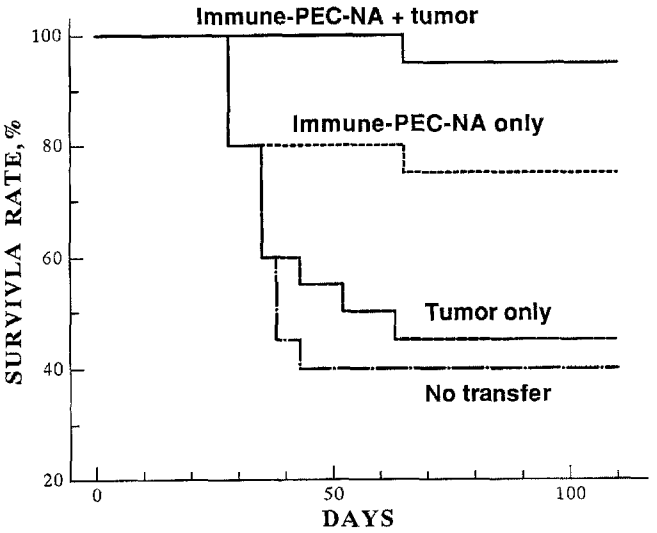

Fig. 1. Effect of adoptive transfer on the prevention of lymph node metastases. A sample of 10^6 MM48 tumor cells was implanted subcutaneously. On days 13 and 14, cyclophosphamide at 70 mg/kg was administered, and on day 14 the tumor-bearing footpad was amputated. On day 15, adoptive transfers were performed in 10–20 mice/group. Significantly increased survival rate in *Immune-PEC-NA + stimulant MM48 tumor* and *Immune-PEC-NA only* groups compared with no treatment or stimulant tumor only ($P < 0.05$, χ^2 test)

Materials and methods

Animals. For all the experiments, specific-pathogen-free (SPF) female inbred C3H/HeSlc mice, 6 weeks of age, were purchased from SLC (Hamamatsu, Japan) and kept under conditions for SPF mice in the facility for experimental animals of our Institute. They were used at 8–10 weeks of age.

Tumors. A transplantable ascites mouse mammary tumor (MM48) cell line was used [25–27]. It was established from a spontaneous mammary tumor of a murine mammary-tumor-virus-positive C3H mouse and has low immunogenicity; MM48 tumor cells take in a normal syngeneic mouse at dose of 100 when inoculated intraperitoneally. A single immunization of 10^6 irradiated MM48 cells provokes little antitumor immunity. Even 10^3 MM48 tumor cells can not be rejected. In order to gain a potent antitumor immunity, a repeated immunization or co-administration of some potent augmenting reagents (e. g. OK-432) is necessary. The acitivity of cytotoxic T cells in the peritoneal exudate cells of repeatedly immunized mice is very low [26]. MM48 tumor cells developed into a solid tumor when an appropriate number of tumor cells were injected subcutaneously and metastasized predominantly to the lymph nodes. As unrelated tumor cells, two syngeneic ascites tumors, mammary tumor FM3A/R and hepatoma MH134 cells, were used.

Reagents used. OK-432, a preparation of streptococcus pyrogens, was provided by Chugai Pharmaceutical Co. Ltd. (Tokyo, Japan). Human recombinant interleukin-2 (IL-2) was a gift from Ajinomoto Co. Ltd. (Tokyo, Japan). Human recombinant interleukin-1 (IL-1) was kindly provided by Otsuka Pharmaceutical Co. Ltd. (Tokyo, Japan).

Preparation of immune lymphocytes. Mice were immunized with 2×10^6 irradiated tumor cells (100 Gy, ^{60}Co source) admixed with 0.1 mg OK-432 once a week for 3 weeks. Between 10 and 11 days after the last immunization, the mice were killed by decapitation and peritoneal exudate cells (PEC) were obtained by lavaging the peritoneal cavity with RPMI-1640 medium containing 7 U/ml heparin. After washing, PEC were passed through a Sephadex G-10 column to obtain a lymphocyte-rich fraction (immune-PEC-NA); 80%–85% of the immune-PEC-NA were $CD4^+$ and there were few $CD8^+$ T lymphocytes.

Preparation of peritoneal macrophage. Mice received an intraperitoneal injection of 0.1 mg OK-432, diluted in phosphate-buffered saline or 1 ml 5% proteose/peptone, and 4 days later they were sacrificed and PEC were obtained. The adherent cell fraction was chosen by incubating the PEC in a petri dish coated with fetal calf serum for 15 min at 37° C. After rinsing out nonadherent cells, the adherent cells were detached by incubating them in 0.2% EDTA at 4° C for 40 min [13]. More than 90% of the adherent PEC phagocytized opsonized sheep erythrocytes. Between 80% and 90% of them were Mac-1^+ and 20%–30% of the OK-432-activated adherent peritoneal cells bore the Ia antigen.

Adoptive transfer. Mice received whole-body irradiation (4 Gy ^{60}Co source), and on the next day 10^4 MM48 tumor cells, with or without 10^6 peritoneal macrophages, were injected subcutaneously into the right hind footpad. On the following day, 5×10^6 immune-PEC-NA (one-mouse equivalent), with or without 2×10^6 irradiated (100 Gy) stimulant tumor cells, were transferred intraperitoneally.

Adoptive immunotherapy for lymph node metastases. A sample of 10^6 MM48 tumor cells was subcutaneously inoculated into the hind footpad. On days 13 and 14, cyclophosphamide (70 mg/kg, Shionogi Pharmaceutical Co. Ltd., Osaka, Japan) was administered intraperitoneally. On day 14, the tumor was resected. As described in our previous report, about 50% of operated mice survived without metastases [27]. In the present experiments, adoptive transfer of immune-PEC-NA with or without stimulant tumor cells was additionally performed on day 15 as described above.

Activation of macrophage by immune-PEC-NA. The immune-PEC-NA alone or immune-PEC-NA plus stimulant tumor cells were inoculated intraperitoneally into the mice that had received intraperitoneal administration of 1 ml 5% proteose/peptone 3 days previously. On the next day, the mice were killed and their peritoneal adherent cells were assayed for cytotoxicity against MM48 and FM3A/R target cells in an ^{125}I-release assay [21]. Target cells were labeled with ^{125}I-deoxyuridine (Amersham Japan, Tokyo, Japan), mixed with effector cells at an E/T ratio of 20 in a 96-well culture plate and cultured for 24 h. Samples were harvested by transferring 0.1 ml supernatant, and their radioactivities were counted. The percentage of cytotoxicity for each assay was calculated by the following formula: cytotoxicity (%) = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] $\times 100$. The range of spontaneous release from the targets was less than 30% of the total isotope count. No cytotoxic activity was detected in the peritoneal macrophages of mice that received 1 ml 5% proteose/peptone only.

Results

Effect of stimulant tumor cells in adoptive transfer of immune-PEC-NA on lymph node metastasis

As reported previously, primary tumor resection alone soon after the appearance of suppressor T cell activity in the regional lymph node did not improve the survival rate of the mice, which eventually died of extensive lymph node metastases, but administration of cyclophosphamide followed by surgical removal of the primary tumor helped to prevent the secondary spread of tumor cells in half of the mice [27]. In this study, in an attempt to improve the survival rate of the other half of the mice that died of metastasis, an adoptive transfer of immune-PEC-NA was further added to this therapeutic regimen 1 day after local tumor resection (Fig. 1). By this treatment, the survival rate was significantly increased in this study (75% on day 100

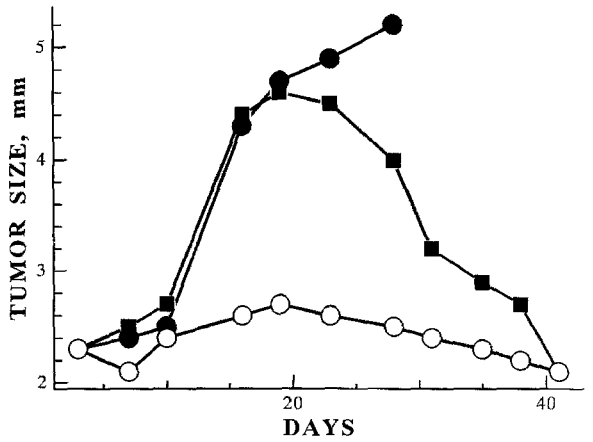

Fig. 2. Effect of stimulant tumor cells added to the adoptive transfer of immune-PEC-NA on the growth of 1-day MM48 tumor. MM48 tumor cells (10^4) were mixed with peritoneal macrophages (10^6) activated by OK-432 and were implanted subcutaneously into the hind foodpad of sublethally irradiated mice. On the next day, immune-PEC-NA (5×10^6) only (■), immune-PEC-NA admixed with irradiated MM48 tumor cells (○), or irradiated tumor cells (2×10^6) only (●) were intraperitoneally transferred into three different groups of mice. Each group consisted of 5 mice. Significant (P <0.05, Student t-test) between ○ and ●; not significant between ■ and ● after 16 days of tumor growth

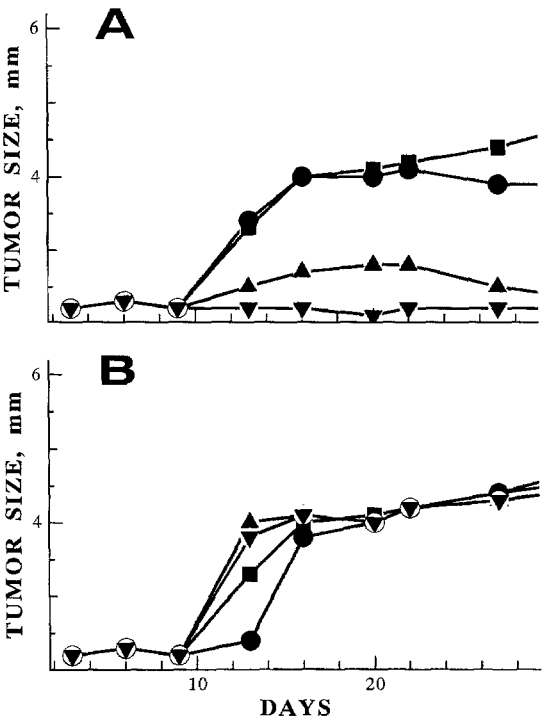

Fig. 3 A, B. Effect of low-dose irradiation of immune-PEC-NA on their antitumor activity in adoptive transfer. Immune-PEC-NA received 5 Gy γ irradiation (^{60}Co) before transfer. **A** Intact immune-PEC-NA. **B** Immune-PEC-NA received 5 Gy irradiation. ●, No transfer; ■, immune-PEC-NA (5×10^6) only; ▲, ten-fold immune-PEC-NA (5×10^7); ▼, immune-PEC-NA (5×10^6) and stimulant tumor cells (2×10^5). Significant (P <0.05, Student t-test) between ▲, ▼ and ●, but not significant between ■ and ● in panel **A**; not significant among four groups in **B**. Five mice were used per group

after adoptive immunotherapy). However, an adoptive transfer of immune-PEC-NA admixed with irradiated MM48 tumor cells led to complete protection against lymph node metastasis in 19 of 20 mice. No significant additional effect was observed when stimulant tumor cells were administered alone or when adoptively transferred immune-PEC-NA were administered with unrelated stimulant tumor cells (data not shown).

Effect of stimulant tumor cells in adoptive transfer of immune-PEC-NA on the growth of 1-day tumor

In this MM48 tumor model, it has previously been demonstrated that antitumor activity in immune-PEC-NA resides in CD4+ T cells. Thus macrophages activated by CD4+ T cells were considered as effectors for eradication of tumor cells. To analyze the efficacy of the admixed transfer of immune-PEC-NA with the relevant tumor stimulant on local tumor growth and the subsequent development of metastasis, mice were whole-body irradiated and, on the next day, viable MM48 tumor cells were subcutaneously inoculated in the footpad after admixture with activated macrophages. Fifteen mice were separated into three groups. The first group received 5×10^6 immune-PEC-NA only, the second received an equal number of immune-PEC-NA admixed with 2×10^6 irradiated MM48 tumor cells, and the last group received only the irradiated tumor cells (Fig. 2).

Tumors in mice that had received only immune-PEC-NA (the first group) initially grew well as control tumors, but around 2 weeks later, the tumor size began to decline. Finally, the tumor in the footpad disappeared in half of the mice, but metastases to the lymph nodes emerged and almost all the mice died. On the other hand, tumor growth in the mice that received the mixture of immune-PEC-NA plus irradiated tumor cells (the second group) was slow and hard to measure, and all mice survived without any sign of lymph node metastasis. This antitumor activity was found to be abrogated by pretreatment of the immune-PEC-NA with anti-CD4 antibody plus complement, but not by pretreatment with anti-CD8 plus complement (data not shown). No difference in tumor growth was observed between mice that received only irradiated MM48 tumor cells (the third group) and mice that received no transfer.

Effect of irradiation on immune-PEC-NA

As shown in Fig. 3, irradiation of immune-PEC-NA (5 Gy) completely abolished their antitumor activity. In the experiments using intact immune-PEC-NA, administration of 5×10^6 immune-PEC-NA alone showed no effect. Administration of 5×10^7 immune-PEC-NA had some degree of antitumor effect but less than that seen in the transfer of immune-PEC-NA plus stimulant tumor cells. An admixture transfer of 5×10^6 immune-PEC-NA with stimulant tumor cells was most effective.

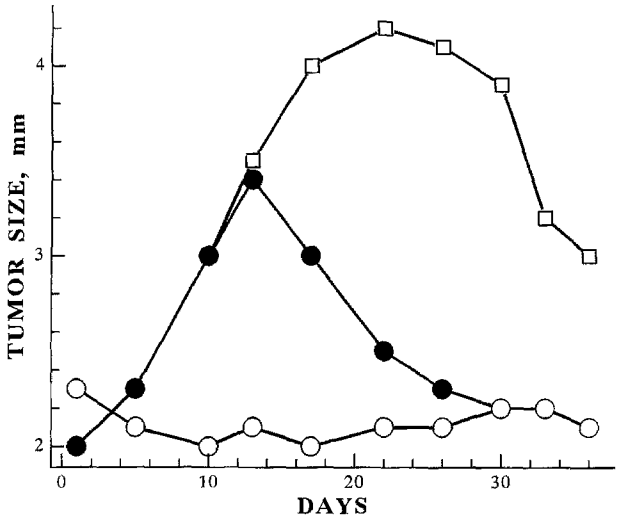

Fig. 4. Effect of activating states of macrophages admixed with implanted tumor cells on the efficacy of adoptive transfer. Two types of peritoneal macrophages were admixed with implanted tumor cells. On the next day, immune-PEC-NA plus stimulant were transferred. ○, Macrophages induced by OK-432; ●, macrophages induced by proteose peptone; □, no macrophages. Significant (P <0.05, Student t-test) between ○, ● and □ after 17 days. Ten mice were used per group

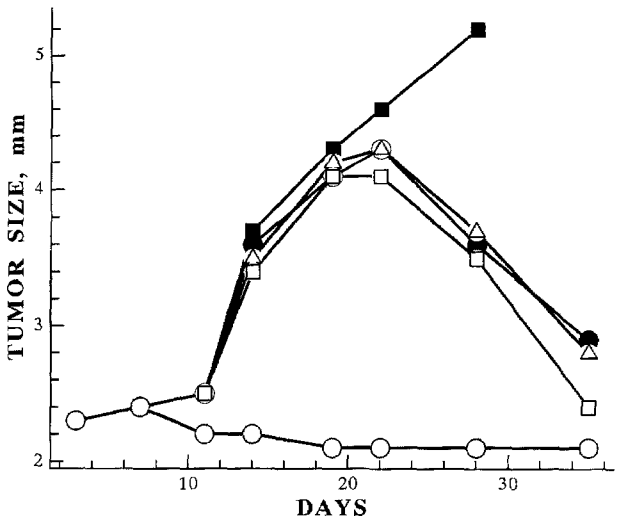

Fig. 5. Tumor specificity of adoptive transfer. Adoptive transfer was performed using irradiated MM48 or other tumor cells as stimulants. MM48 tumor cells were implanted with macrophages activated by OK-432. Immune-PEC-NA against MM48 were admixed with irradiated MM48 (○), FM3A/R (△), MH134 (□), no tumor (●) and were transferred intraperitoneally. Immune-PEC-NA against FM3A/R were admixed with irradiated FM3A/R and transferred into MM48-tumor-bearing mice (■). Significant (P <0.05, Student t-test) between ○ and ●; not significant between △, □, ■ and ●. Five mice were used per group

Effect of the activating state of macrophages within a local tumor on the therapeutic efficacy of the adoptive transfer system

In the above experiments, tumor cells were implanted after admixture with peritoneal macrophages induced by OK-432 (OK-432 macrophages). The OK-432 macrophages showed non-specific cytotoxicity against not only MM48 and FM3A/R but also against MH134 tumor cells in a ^{51}Cr-release assay in vitro, while the peritoneal macrophages induced by proteose/peptone did not show any cytocidal activity for any of the tumor cells used (data not shown). However, both types of macrophages showed little neutralizing activity in vivo. These two types of macrophages were compared for their therapeutic efficacy in the same adoptive transfer system (Fig. 4). The resultant antitumor effects were observed in the following order: no macrophages < proteose/peptone-elicited macrophages < OK-432 macrophages. Histological examination of metastases to the regional (popliteal) lymph nodes showed the same tendency. The group treated with OK-432 macrophages showed no metastasis, the group that received proteose/peptone macrophages showed metastasis in 2 of 10 mice, and in the group that received no macrophages, metastases developed in all the mice.

Tumor specificity of immune-PEC-NA

To determine the specificity of the admixed stimulant tumor cells, immune-PEC-NA collected from MM48-immunized mice were admixed with irradiated MM48, FM3A/R or MH134 tumors and were transferred into the peritoneal cavity of MM48-bearing mice (Fig. 5). Irrelevant tumors, MH134 or FM3A/R, used as stimulant tumors, showed no stimulating effect, comparable to that of immune-PEC-NA alone. In addition, adoptive transfer of immune-PEC-NA against FM3A/R admixed with stimulant FM3A/R tumor cells showed no antitumor effect on MM48 tumor growth. These results indicate that there is a strict tumor specificity required among the immunizing tumor, the stimulant tumor and the target tumor for efficient eradication of tumor cells. Even without stimulant tumor cells, immune-PEC-NA showed tumor specificity. Immune-PEC-NA against MM48 did not show any antitumor effects on the growth of FM3A/R and vice versa.

Next we examined the possibility of innocent-bystander lysis of tumor cells. Mice were inoculated with different tumors or a tumor mixture of MM48 and FM3A/R into the right and left hind footpads, and this was followed by adoptive transfer of immune-PEC-NA against MM48 1 day later (Fig. 6). As a result, the growth of MM48 was inhibited, but the tumor mixture and FM3A/R grew well. There was no significant difference in the growth rate between FM3A/R and the tumor mixture. The growing tumor within the tumor mixture was revealed to be FM3A/R, as determined with a specific antibody (data not shown), indicating that no definite innocent-bystander tumor lysis takes place and that activated macrophages do not function as well as presumed to eliminate tumor cells.

Administration of IL-2 instead of stimulant tumor cells

Antigenic stimulation for the clonal expansion of immune T cells in vivo seems to be essential for the effectiveness of the immunological eradication of tumor cells (Figs. 2, 3).

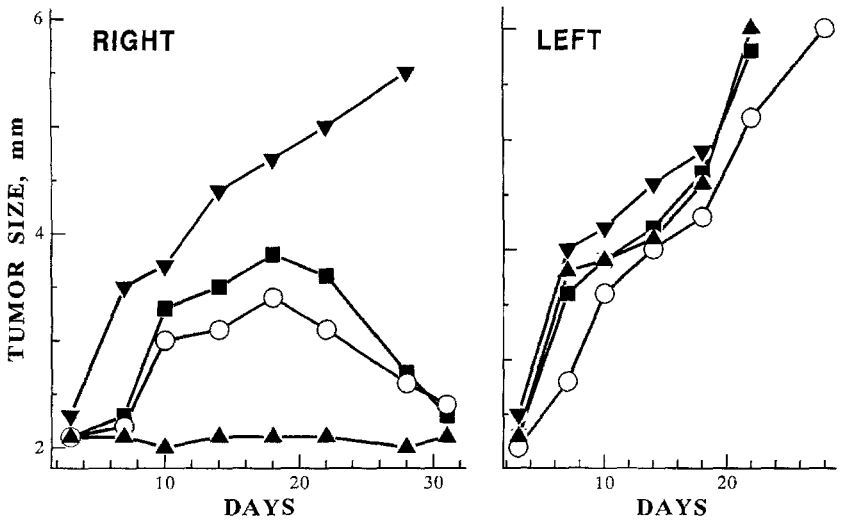

Fig. 6. Absence of innocent-bystander lysis. Mice were inoculated with different tumor cells into the right (Rt) and left (Lt) footpads. ○, Rt MM48 and Lt FM3A/R; ▲, Rt no tumor and Lt mixture of MM48 and FM3A/R; ■, Rt MM48 and Lt tumor mixture; ▼, Rt FM3A/R and Lt tumor mixture. Adoptive immunotherapy against MM48 was carried out in these groups. Five mice were used per group

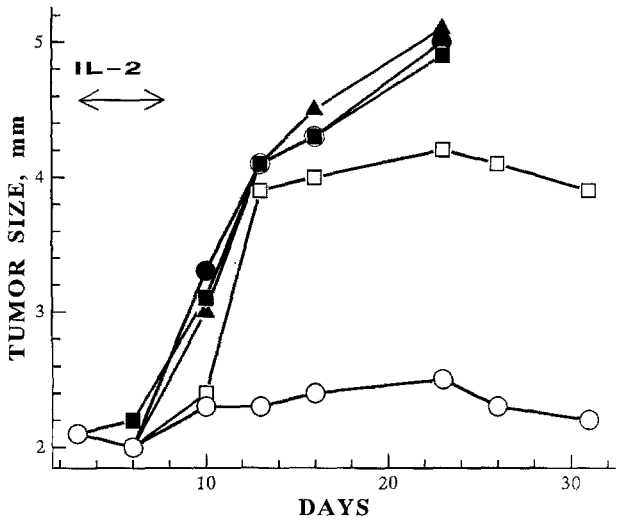

Fig. 7. Effect of exogenous administration of interleukin-2 (IL-2). Adoptive transfers were performed as described in Materials and methods. Human recombinant IL-2 was administered intraperitoneally twice a day for 7 days beginning on the day of adoptive transfer. ●, No transfer; □, immune-PEC-NA only; ○, immune-PEC-NA plus stimulant; ■, immune-PEC-NA plus IL-2 (250 U × 2/day); ▲, immune-PEC-NA plus IL-2 (25000 U × 2/day). Significant (P <0.05, Student t-test) between ○ and ●; not significant between, □, ■, ▲, and ●. ◄—►, Duration of cytokine administration. Five mice were used per group

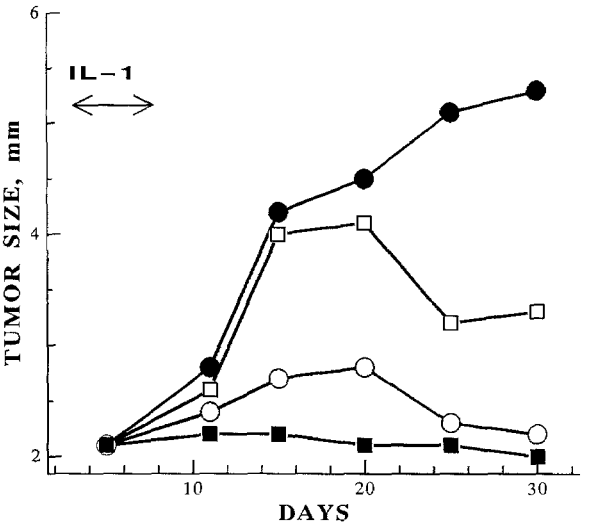

Fig. 8. Effect of concomitant administration of IL-1. Adoptive transfers were performed as described in Materials and methods. Human recombinat IL-1 (250 U/mouse) was intraperitoneally given twice a day for 7 days beginning on the day of adoptive transfer. ●, No transfer; □, immune-PEC-NA only; ○, immune-PEC-NA plus stimulant; ■, immune-PEC-NA plus IL-1. Significant (P <0.05, Student t-test) between ○, ■ and ●; not significant between □ and ●. ◄—►, Duration of cytokine administration. Five mice were used per group

Recombinant interleukin-2 (IL-2), a potent T cell growth factor, was concomitantly given with immune-PEC-NA instead of stimulant tumor cells, and antitumor effects were compared. IL-2 at a dose of 25000 U or 250 U/mouse was given twice a day for a week beginning from the day of adoptive transfer (Fig. 7). IL-2 did not confer any potentiation of antitumor activity. Surprisingly, in mice given IL-2, tumors grew as well as in control mice (no transfer), and no sign of tumor regression was observed in spite of immune-PEC-NA administration. Thus, additional administration of IL-2 abrogated the constitutive antitumor activity of immune-PEC-NA.

Administration of IL-1 instead of stimulant tumor cells

On the other hand, adoptive transfer of immune-PEC-NA with concomitant administration of interleukin-1 (IL-1) at 250 U/mouse induced more potent antitumor activity than that with stimulant tumor cells (Fig. 8). IL-1 at 2500 U/mouse showed less effect and 25 U/mouse had no effect on therapeutic efficacy (data not shown). Administration of IL-1 alone showed no effect on tumor growth at any dose.

In the adoptive transfer of immune CD4+ T cells with exogenous IL-1, the effect of activated macrophages ad-

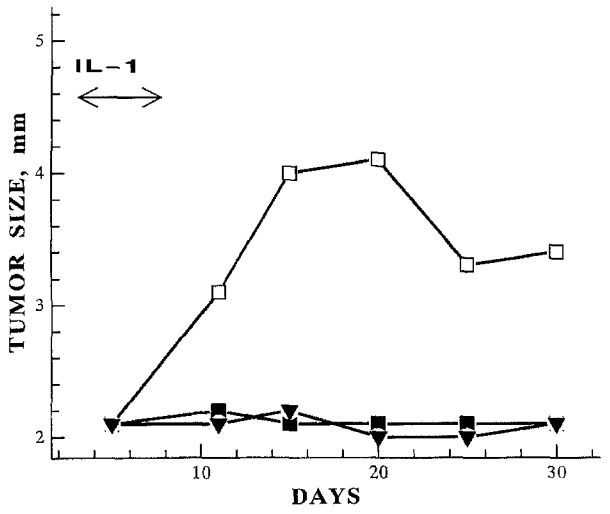

Fig. 9. Effect of activating state of macrophages admixed with implanted tumor cells on the efficacy of adoptive transfer of immune-PEC-NA and exogenous IL-1. Tumor cells alone (▼) or tumor cells admixed with macrophages induced by OK-432 (■) were inoculated. On the next day, immune-PEC-NA was transferred. Exogenous IL-1 (250 U/mouse, twice a day) was given from the day of transfer for 1 week. □, Macrophages induced by OK-432 (IL-1 was not administered). Significant ($P < 0.05$, Student t-test) between ■, ▼ and □; not significant between ■ and ▼. ◄—►, Duration of cytokine administration. Five mice were used per group

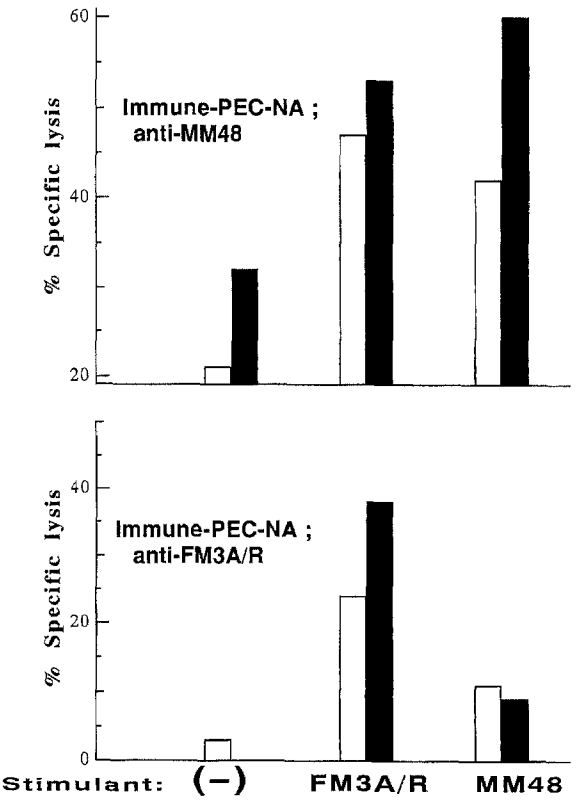

Fig. 10. Cytotoxicity of peritoneal macrophages activated by immune-PEC-NA and stimulants. Naive mice received a 1-ml intraperitoneal injection of 5% proteose/peptone, and 3 days later immune-PEC-NA (5×10^6) only or immune-PEC-NA plus stimulants were transferred. On the next day, peritoneal macrophages were assayed for cytotoxicity against ^{125}I-labeled tumor cells. *Open bar*, MM48 as target; *closed bar*, FM3A/R as target. ^{125}I-release assay, E/T ratio = 20/1, 24-h incubation. *Abscissa*, stimulant tumor cells, which were admixed with immune-PEC-NA and intraperitoneally transferred into the mice for activation of peritoneal macrophages

mixed with the inoculated tumor was examined (Fig. 9). Implanted tumor cells failed to grow irrespective of the presence or absence of OK-432 macrophages at the tumor site when immune CD4+ T were adoptively transferred with IL-1 administration. Thus it is obvious that the pre-accumulation or pre-activation of macrophages surrounding tumor cells is not necessary for eradication of tumor by adoptive transfer of immune CD4+ T cells plus administration of IL-1.

Activation of peritoneal macrophages by CD4+ T cells

To check the specificity of macrophages activated by CD4+ T cells, the peritoneal macrophages were activated by inoculating immune-PEC-NA against MM48 or FM3A/R with the relevant irradiated tumor cells (Fig. 10). The cytotoxic cells were Mac-1+ adherent cells and lost their cytotoxic capacity after incubation with carageenan, but not after incubation with anti-Thy1 antibody plus complement (data not shown). Unexpectedly immune-PEC-NA, obtained by immunizing mice with MM48, evoked macrophage activation after inoculation not only with MM48 but also with FM3A/R or even with no stimulant tumor. On the other hand, specific T cells against FM3A/R activated macrophages only when inoculated with FM3A/R. However, the tumoricidal macrophages activated by both types of tumor-specific T cells showed cytotoxicity nonspecifically against both MM48 and FM3A/R, just as did the macrophages activated by non-specific macrophage activators, e.g. OK-432 (data not shown).

Discussion

In this study, we succeeded in the eradication of secondary tumor spread by adoptive transfer of tumor-specific CD4+ T cells plus irradiated tumor cells 1 day after administration of cyclophosphamide and surgical removal of established local tumor. In our previous experiments, we observed that the induction of tumor-specific CD4+ regulatory T cells in the regional lymph nodes of mice bearing subcutaneous MM48 tumor was associated with the retardation of local and metastatic tumor growth and that the emergence of CD8+ cytotoxic T cells was only seen in the regional lymph nodes after the complete recovery of mice from metastasis or in immune-PEC-NA of mice immunized intraperitoneally with irradiated tumor cells [25–27]. However, the activities of both T cell populations were very weak without the use of a potent biological response modifier such as OK-432, a streptococcal preparation. The mice immunized with a combination of tumor cells and OK-432 showed three times as many T cells as were in PEC of mice immunized with tumor cells alone, and rejected a tumor load ten times as great as that rejected

by mice immunized with tumor cells alone when viable tumor cells were challenged intraperitoneally. But administration of OK-432 without tumor cells did not induce any potent CD4+ T cells in the peritoneal cavity. OK-432 is one of the potent interferon inducers [23]. Early induction of interferon by OK-432 may confer a successful induction of specific immunities [11] in those models.

In order to analyze the mechanism of adoptive immunity obtained with immune CD4+ T cells plus a relevant tumor stimulant, by which metastatic tumor cells were eradicated after the removal of the primary tumor, we used 1-day tumor with macrophages that were inoculated subcutaneously into the hind footpad of whole-body-irradiated mice. Enhanced antitumor activity of CD4+ T cells was observed in tumor-bearing mice after the adoptive transfer of an admixture of immune T cells with relevant tumor cells (Fig. 2). The immune T cells were very sensitive to irradiation (Fig. 3), and to succeed in the curative eradication of metastatic tumor cells in lymph nodes, the strict identification of tumor-antigen specificity was required among the target tumor, tumor for immunization and stimulant tumor (data not shown), similar to the data depicted in Fig. 5. These results show that antigenic stimulation and subsequent proliferation of tumor-specific CD4+ T cells contribute to the enhancement of immunotherapy [4, 8]. However, administration of IL-2, a potent T-cell growth factor, abolished even the constitutive antitumor activity of immune-PEC-NA, resulting in an unfavorable effect on the host (Fig. 7). The immune CD4+ T cells possessed IL-2 receptors, as determined by 3C7 monoclonal antibody [22] (data not shown). IL-2 induced marked proliferation of immune-PEC-NA, while IL-1 caused no such remarkable effect in vitro (data not shown). We have no data to explain why IL-2 does not work in this immunotherapy, but it may be assumed that continued antigenic stimulation of CD4+ T cells is necessary for the successful induction of antitumor immunity, and the nonspecific proliferation of T cells by IL-2 administration is detrimental of therapeutic efficacy.

There are two CD4+ T cell populations, Th1 and Th2 [1, 2, 14, 18]. Th1 cells secrete interferon γ (IFNγ), lymphotoxin and IL-2 upon antigenic stimulation and mediate cellular immunity, including delayed-type hypersensitivity, to participate in antitumor immunity. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, which facilitate the development of humoral immunity [24]. Recently it was shown that IFNγ and IL-10, respectively synthesized by Th1 and Th2 cells, cross-regulate the function of both CD4+ T cell populations. IFNγ inhibits the proliferation of Th2 cells and IL-10 inhibits cytokine synthesis of Th1. On the other hand, IL-2 and IL-4 up-regulate the responses of both Th1 and Th2 cells [24]. Therefore, it may be possible to induce the suppression of cellular immunity by systemic administration of IL-2, providing that tumor-specific Th1 cells are a minority of the CD4+ T cell population. This hypothesis might be compatible with the previous data that the tumor-specific suppressor T cells that emerged in the lymph node just before the establishment of metastasis were also CD4+ [26].

Regarding the recruitment of immune T cells, one possible reason for the ineffectiveness of the adoptive transfer by IL-2 administration may be related to the early entrapment of lymphocytes by the reticuloendothelial system, as it has been reported that splenocytes cultured in T-cell growth factor showed earlier increased traffic to the lung than did uncultured splenocytes [15, 16].

It is well known that non-cytotoxic and tumor-specific CD4+ T cells activate macrophages to eradicate tumor cells in vivo [7, 19]. In this study, immune-PEC-NA were able to stimulate macrophages to induce tumoricidal activity (Fig. 10). But if the cytocidal macrophages do work in tumor destruction in vivo, both MM48 and FM3A/R tumors should be eliminated in a non-specific fashion. This was not the case in the MM48 tumor model (Fig. 6). In addition, adoptive transfer of immune-PEC-NA against FM3A/R plus irradiated FM3A/R has resulted in a less significant antitumor effect against MM48, if any (Figs. 5 and 10). Of course, the possibility is not excluded that delayed-type hypersentitivity does take place in this response, but it is less probable that the activated macrophages are the final effectors for tumor destruction, because innocent-bystander lysis of a different antigenic FM3A/R adjacent to MM48 did not take place (Fig. 6). This evidence indicates that specific antigen recognition is necessary in the last stage of tumor destruction. It can be assumed, therefore, that the final tumor destruction is predominantly mediated by a cell population equipped with antigen receptor, probably cytotoxic T cells.

It is also well known that macrophages have the other important role as accessory/antigen-presenting cells in the expression of antitumor functions of tumor-specific CD4+ T cells. In this study, the activating states of the macrophages inoculated with tumor cells into the hind footpad showed considerable relevance to the implementation of intraperitoneal transfer (Fig. 4). Thus, it is quite reasonable that the macrophages serve as accessory cells to secrete IL-1 for the proliferative responses of CD8+ T cells to the tumor, since the addition of exogenous recombinant IL-1 was able to substitute for macrophages in this response and surpassed the activating state of macrophages [12]. Macrophages induced by OK-432 secrete a larger amount of IL-1 [10]. It is conceivable that exogenous IL-1 can not only take over the role of stimulant tumor but also reinforce the antitumor activity of CD8+ T cells mediated by CD4+ T cells to eliminate local and metastatic tumors. Furthermore, IL-1 may enhance the immunogenicity of poorly immunogenic tumors [17] and may also promote tumor eradication by augumenting the antitumor response of helper and cytotoxic T cells [3, 5, 20]. In the case of murine leukemia, combined IL-1 and IL-2 therapy potentiated host antitumor activity to eradicate metastatic tumor cells, and CD8+ T cells were the final effector for tumor destruction [3].

In term of adoptive immunotherapy for lymph node metastasis, it must be stressed that the timing of combined cancer treatments is of importance. We administered the treatments at the time when tumor-specific suppressor T cells had just appeared in the regional lymph nodes of mice bearing established tumors. However, when the treatment was begun 2 days later, it failed to eradicate metastatic tumor cells. Thus, further investigations are needed to improve the therapeutic efficacy of anti-metastatic activity.

References

1. Cher DJ, Mosmann TR (1987) Two types of murine helper T cell clone: II. Delayed-type hypersensitivity is mediated by Th1 clones. J Immunol 138: 3688
2. Cherwinsky HM, Schumacher JH, Brown KD, Mosmann TR (1987) Two types of helper T cell clone: III. Further differences in lymphokine synthesis between Th1 and Th2 clones revealed by RNA hybridization, functionally monospecific bioassays, and monoclonal antibodies. J Exp Med 166: 1229
3. Cioli V, Gabriele L, Sestili P, Varano F, Proietti E, Gresser I, Testa U, Montesoro E, Bulgarini D, Mariani G, Peschele E, Belardelli F (1991) Combined interleukin 1/interleukin 2 therapy of mice injected with highly metastatic Friend leukemia cells: host antitumor mechanisms and marked effect on established metastases. J Exp Med 173: 313
4. Colombo MP, Parenza M, Parmiani G (1985) Adoptive immunotherapy of a BALB/c lymphoma by syngeneic anti-DBA/2 immune lymphoid cells: characterization of the effector population and evidence for the role of the host's non-T cells. Cancer Immunol Immunother 20: 198
5. Ebina T, Murata K (1991) Antitumor effector mechanism of interleukin-1β at a distant site in the double graft tumor system. Jpn J Cancer Res 82: 1292
6. Fujiwara H, Fukuzawa M, Yoshioka T, Nakajima H, Hamaoka T (1984) The role of tumor-specific Lyt-1$^+$2$^-$ T cells in eradicating tumor cells in vivo: I. Lyt-1$^+$2$^-$ T cells do not necessarily require recruitment of host's cytotoxic T cell precursors for implementation of in vivo immunity. J Immunol 133: 1671
7. Fujiwara H, Takai Y, Sakamoto K, Hamaoka T (1985) The mechanism of tumor growth inhibition by tumor-specific Lyt-1$^+$2$^-$ T cells: I. Antitumor effect of Lyt-1$^+$2$^-$ T cells depends on the existence of adherent cells. J Immunol 135: 2187
8. Greenberg PD, Cheever M, Fefer A (1979) Suppression of the in vitro secondary response to syngeneic tumor and of in vivo tumor therapy with immune cells by culture-induced suppressor cells. J Immunol 123: 515
9. Greenberg HP, Cheever MA, Fefer A (1981) Eradication of disseminated murine leukemia by chemoimmunotherapy with cyclophosphamide and adoptively transferred immune syngeneic Lyt-1$^+$2$^-$ lymphocytes. J Exp Med 154: 952
10. Ichimura O, Suzuki S, Sugawara Y, Osawa T (1983) Lymhokine induction by streptococcal preparation, OK-432 (PICIBANIL). In: Proceedings of 13th International Congress of Chemotherapy. Egermann
11. Ishikawa S, Tachibana T (1986) Killer-helper effect of L cells in the generation of anti-MM cytotoxic T lymphocytes: replacement by beta-interferon and mechanism of action. Jpn J Cancer Res 77: 931
12. Kern DE, Peace DJ, Klarnet JP, Cheever MA, Greenberg PD (1986) Requirement for recognition of class II molecules and processed tumor antigen for optimal generation of syngeneic tumor-specific class I-restricted CTL. J Immunol 136: 4303
13. Kumagai K, Itoh K, Hinuma S, Tada M (1979) Preparation of plastic Petri dishes with fetal calf serum. A simple method for macrophage isolation. J Immunol Methods 29: 19
14. Kurt-Jones EA, Hamberg S, Ohara J, Paul WP, Abbas AK (1987) Hetergeneity of helper/inducer T lymphocytes: I. Lymphokine production and lymphokine responsiveness. J Exp Med 166: 1774
15. Lotze MT, Line BR, Mathisen DJ, Rosenberg SA (1980) The in vivo distribution of autologous human and murine lymphoid cells grown in T cell growth factor (TCGF): implications for the adoptive immunotherapy of tumors. J Immunol 125: 1487
16. Mathisen DJ, Rosenberg SA (1980) The in vivo distribution of transferred syngeneic, allogeneic, and xenogeneic lymphoid cells: implication for the adoptive immunotherapy of tumors. J Immunol 124: 2295
17. McCune CS, Marquis DM (1990) Interleukin 1 as an adjuvant for active specific immunotherapy in a murine tumor model. Cancer Res 50: 1212
18. Mosmann TR, Cherwinsky H, Bond MW, Giedlin MA, Coffman RL (1986) Two types of murine helper T cell clone: I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol 136: 2348
19. Nakajima H, Fujiwara H, Takai Y, Izumi Y, Sano S, Tuchida T, Hamaoka T (1985) Studies on macrophage-activating factors (MAF) in antitumor immune responses: I. Tumor-specific Lyt-1$^+$2$^-$ T cells are required for producing MAF able to generate cytolytic as well as cytostatic macrophages. J Immunol 135: 2199
20. North RJ, Neubauer RH, Huang JJH, Newton RC, Loveless SE (1988) Interleukin-1-induced T cell-mediated regression of immunogeneic murine tumors. Requirement for an adequate level of already acquired host concomitant immunity. J Exp Med 168: 2031
21. Okuda T, Tachibana T (1988) Involvement of Mac-1 antigen in tumor cell killing by macrophages. Tohoku J Exp Med 155: 211
22. Ortega GR, Robb RJ, Shevach EM, Malek TR (1984) The murine IL-2 receptor: I. Monoclonal antibodies that define distinct functional epitopes on activated T cells and react with activated B cells. J Immunol 133: 1970
23. Saito M, Ebina T, Koi M, Yamaguchi T, Kawade Y, Ishida N (1982) Induction of interferon-γ in mouse spleen by OK-432, a preparation of streptococcus pyrogens. Cell Immunol 68: 187
24. Street NE, Mosmann TR (1991) Functional diversity of T lymphocytes due to secretion of different cytokine patterns. FASEB J 5: 171
25. Yoshida K, Tachibana T (1985) The primary immunoregulatory role of regional lymph node on the development of local tumor and lymphatic metastasis. In: Basic mechanisms and clinical treatment of tumor metastases. Academic Press, New York, p 335
26. Yoshida K, Tachibana T (1985) Studies on lymphatic metastasis: I. Primary immunoregulatory role of regional lymph node in the establishment of lymphatic metastasis. JNCI 75: 1049
27. Yoshida K, Tachibana T (1990) Cyclophosphamide-dependent lymph node modification in lymph node metastasis of MM48 tumor cells in syngeneic mice. Jpn J Cancer Res 81: 253