Acta Medica et Biologica
Vol. 46, No.4, 121-131, 1998

Mechanism of Antitumor Effect Mediated by *In Vitro* Activated Tumor-draining Lymph Node Cells

Yoshie MARUYAMA, Hirohisa YOSHIZAWA and Masaaki ARAKAWA

Department of Internal Medicine (II), Niigata University Medical School, Niigata, Japan

Received July 21 1998; accepted September 7 1998

Summary. **We previously demonstrated that tumor-specific effector cells could be generated from tumor-draining lymph node cells by *in vitro* sequential activation with anti-CD3 mAb and IL-2. In the present study, we further examined cellular interactions and mechanisms of the antitumor effect. Tumor-draining lymph node cells (TDLN) of a weakly immunogenic fibrosarcoma, MCA 205, were activated by the anti-CD3/IL-2 method and transferred into the peritoneal cavities of mice bearing 5-day established peritoneal dissemination of MCA 205 tumor cells. Intraperitoneal injection of anti-CD3/IL-2-activated TDLN significantly prolonged the survival period, and 80% of treated mice remained tumor free for more than 80 days. *In vivo* depletion of host macrophages with $GdCl_3$ clearly abrogated the antitumor effect, while the elimination of host T cells with sublethal irradiation (500 R) did not, indicating that host macrophages are indispensable in the antitumor effect. *In vitro* cytotoxicity and cell proliferation were further examined in detail to determine the commitment of macrophages and mediator cytokines. Although anti-CD3/IL-2 activated cells mediated minimal cytotoxicity in a 4-hr ^{51}Cr releasing assay, tumor specific cytolytic action was detected in an 18-hr assay with coexisting peritoneal cells. The results suggest that peritoneal macrophages play an indispensable role in this treatment effect and that T cell-macrophage interactions were mediated by IFN-γ, the importance of which was exhibited in the early phase of the tumor-effector-macrophage contact.**

Key words—adoptive immunotherapy, peritoneal cavity, macrophages, $GdCl_3$, IFN-γ.

Correspondence: Yoshie Maruyama, Department of Internal Medicine (II), Niigata University Medicine School, Asahimachi 1, Niigata 951-8520, Japan.

INTRODUCTION

Adoptive immunotherapy for cancer has been extensively explored and well documented in various experimental animal models[1,2,3].

We previously demonstrated that tumor draining lymph nodes (TDLN), which are well known to contain an enriched population of precursor lymphocytes of tumor-sensitized T cells, can be activated and expanded by sequential culture with monoclonal antibodies to T-cell receptor complex (anti-CD3 or anti-TCR) and IL-2. These effector T cells were highly effective in the treatment of a lung metastasis model[4,5,6,7]. Stable *in vitro* expansion of effector cells induced by this method allowed us to conduct clinical trials whereby various kinds of cancer patients were treated with anti-CD3/IL-2-activated T cells[8,9]. Although immune effector cells generated in animal models were highly effective *in vivo*, a limited number of cancer patients responded to the therapy. To establish more effective immunotherapy in clinical settings, further investigation to elucidate the process of tumor eradication mediated by immune effector cells has been attempted[9]. However, the precise *in vivo* mechanism(s) by which these immune cells mediate immunological injury to tumors upon adoptive transfer is not clear, and little has been reported on the role of the immune system of the tumor-bearing host, such as the activities of lymphocytes and macrophages in tumor eradication.

To examine the precise *in vivo* antitumor mechanism(s) of immune effector cells, we used peritoneal dissemination models and investigated the roles of host lymphocytes and macrophages on antitumor efficacy mediated by anti-CD3/IL-2-activated tumor-draining lymph node cells. We also investigated the roles of 2 cytokines, IFN-γ and TNF-α,

assumed to regulate this collaborative effector-host component network in tumor eradication. We demonstrate here the indispensable involvement of host macrophages and the importance of IFN-γ, which plays a key role in the early phase of the tumor-effector-macrophage contact in this particular intraperitoneal treatment model.

MATERIALS AND METHODS

Mice

Female C57BL/6 (B6) mice were purchased from the CLER Laboratory (Tokyo, Japan). They were maintained in specific pathogen-free conditions and were used for experiments at the age of 10 weeks or older.

Tumors

The MCA 205 (205) is a 3-methylcholanthrene-induced fibrosarcoma of C57BL/6 (B6) origin[10]. This weakly immunogenic tumor was maintained *in vivo* by serial s. c. transplantation of cryopreserved tumor samples, and was used in its sixth transplantation generation for the current study. Another similarly induced tumor, MCA 203 (203) sarcoma of B6 origin in its sixth transplantation generation, served as a specificity control. Single-cell suspensions were prepared from solid tumors by mincing and then digesting with constant stirring in 40ml Hank's balanced salt solution (HBSS) containing 4 mg DNase, 40 mg collagenase and 100 U of hyaluronidase (Sigma Chemical Co., St Louis, MO, USA) for 3 hr at room temperature. The mixture was poured through a layer of 100 nylon mesh (Nytex; TETKO Inc., Briarcliff Manor, NY, USA) and washed twice with HBSS.

Anti-CD3 monoclonal antibody (mAb)

A rat mAb, YCD-3, directed against the CD3-ε chain of the murine TCR/CD3 complex was kindly provided by Dr. Kim Bottomly, Yale University. The YCD-3 mAb was harvested as a supernatant of *in vitro* culture with the hybridoma cells, and then partially purified by 50% ammonium sulfate precipitation, and the IgG content was determined by enzyme-linked immunosorbent assay (ELISA).

Recombinant IL-2

Human recombinant interleukin 2 (rIL-2) was kindly supplied by the Takeda Co. (Osaka, Japan). The biological and biochemical activities of this material have been described elsewhere[11]. Purified material had a specific activity of 3.6×10^6 U/mg. In this report, all data are expressed in BRM units.

Tumor-draining lymph nodes (TDLN)

B6 mice were inoculated s. c. to the bilateral flank with 2×10^6 viable MCA 205 tumor cells suspended in 0.05 ml HBSS. Ten to 12 days later, when the size of the flank tumor reached approximately 8-10 mm, inguinal TDLN were removed aseptically, and lymphocyte single cell suspensions were prepared by teasing LN with 20-G needles and then pressing with the blunt end of a plastic syringe in HBSS.

Activation of LN cells with anti-CD3/IL-2

TDLN cells, $1\text{-}1.6\times10^8$, were incubated in 30 ml of conditioned medium (CM) with 20% anti-CD3 mAb supernatant in a 75-cm^2 tissue culture flask for 2 days at 37℃ in 5% CO_2 atmosphere. After washing, the recovered cells were expanded in 40 U/ml of IL-2 in a 163-cm^2 culture flask for 3 days. CM consisted of RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM fresh L-glutamine, 100 mg streptomycin, 100 U penicillin, 50 mg gentamicin, 0.5 mg fungizone/ml (all from Life Technologies, Gaithersburg, MD) and 5×10^{-5} M 2-mercaptoethanol (Sigma Chemical Co. St. Louis, MO, USA). These anti-CD3/IL-2-activated cells were then harvested, washed, and resuspended in HBSS for adoptive immunotherapy, or in CM for *in vitro* assay.

Adoptive immunotherapy protocol

Peritoneal dissemination of 205 tumor was initiated by intraperitoneal inoculation (i. p.) of B6 mice with 2×10^6 tumor cell suspension in 1 ml of HBSS. Five days later, 2×10^6 of anti-CD3/IL-2-activated TDLN cells (CD3-AT cells) or lymphokine-activated killer (LAK) cells were adoptively transferred (AT) to the intraperitoneal cavity of each mouse. Therapeutic efficacy was evaluated by the survival of treated animals, and the significance of differences in survival periods between groups was calculated by a generalized Wilcoxon test.

Depletion of host lymphocytes

Mice were given 500 rads whole-body irradiation 1 day prior to tumor i. p. inoculation. This dose of

radiation was sublethal and enough to suppress radiosensitive T-cell components but not the macrophages of the mice.

Depletion of host macrophages

Mice were given $GdCl_3$ i. v. 1 mg/0.5 ml HBSS administration 1 day prior to tumor i. p. inoculation. The $GdCl_3$ was purchased from Wako Chemical Co. (Tokyo, Japan) and has been shown to suppress Kupfer cells in mouse liver[12].

In vitro cytotoxicity assay

Four-hour and 18-hr ^{51}Cr release assays were used to determine the cytotoxic activity of anti-CD3/IL-2 activated cells. The 205 and 203 tumor cells (10^7) were labeled with $Na^{51}Cr^4$, 100μCi, (Daiichi Radio Isotopes, Tokyo, Japan) at 37℃ for 1 hr and washed 3 times in CM. Target cells (10^4) were incubated with effector cells at 37℃ in a volume of 0.2 ml CM for 4 or 18 hr. The supernatant was collected, and the samples were counted in a γ-counter. The percent lysis was calculated as follows:

$$\%\text{lysis}=\frac{\text{experimental cpm}-\text{spontaneous cpm}}{\text{maximum cpm}-\text{spontaneous cpm}}\times 100\ (\%)$$

Flow cytometric analysis

Analysis of cell surface markers was carried out by indirect immunofluorescence. Stained cell preparations were analyzed in a fluorescence-activated cell sorter (FACS) scan flow microfluorometer (Becton Dickinson, Sunnyvale, CA, USA). Five to 10×10^5 cells were incubated for 45 min. at 4℃ with 25 ml of diluted mAb in phenol red-free HBSS containing 2% FCS and 0.1% NaN_3. Bound antibodies were detected by incubation with 20 μl FITC-labeled rabbit anti-rat lgG or goat anti-hamster (Caltag Laboratories, San Francisco, CA, USA), depending on the species of primary mAb used. Fluorescence profiles were generated by analyzing 10,000 cells, and displayed as logarithmically increasing fluorescence intensity versus cell numbers.

In vitro cell proliferation and cytokine release assays

Two$\times10^4$ 205 cells were admixed with 2 $\times10^5$ CD3-AT cells generated from MCA 205 TDLN and were cultured in 2 ml complete medium in 24-well flat bottom plates (Costar). Peritoneal exudate cells (PEC) were collected from mice 3 days after tumor i. p. inoculation, and 4 $\times10^4$ PEC were added to assigned wells. The number of cells was counted on days 1 to 7, and culture supernatants were collected and tested in duplicate in IFN-γ and TNF-α enzyme-linked immunosorbent assays (Genzyme, Cambridge, MA, USA). For *in vitro* blocking experiments, 25 μg of either anti-IFN-γ or anti-TNF-α antibody were added to the assigned wells. Hybridoma (R4-6A2) producing anti-IFN-γ mAb was obtained from the American Type Culture Collection, and culture supernatant was partially purified and used for the experiments. This antibody has been shown to neutralize IFN-γ but not IFN-α or-β[13]. Polyclonal rabbit anti-mouse TNF-α antibody was purchased from Genzyme Co. (Cambridge, MA, USA), and has been shown to neutralize LPS-induced production of TNF[14].

In vivo antibody blocking experiments

For the *in vivo* blocking experiments, mice in each were given injection of 2×10^6 tumor cells i. p. on day 0. CD3-AT cells were adoptively transferred on day 5. Two days later or concomitant to adoptive transfer of CD3-AT cells, 2.5 mg of either anti-IFN-γ or anti-TNF-α antibody in 0.5 ml HBSS was administered i. p.

RESULTS

Effect of CD3-AT cells on peritoneal tumor dissemination of mice

In this study, we used a peritoneal tumor dissemination model to investigate the antitumor mechanisms of anti-CD3/IL-2-activated TDLN cells (CD3-AT cells).

First, we examined whether CD3-AT cells could prolong the survival of mice bearing peritoneal dissemination of tumors. As shown in Fig. 1, mice receiving no treatment or IL-2 only died within 35 days of tumor inoculation into the peritoneal cavity. In contrast, mice receiving CD3-AT cells, with or without IL-2 administration, mostly survived over 120 days, and 10 of 11 mice were cured of disease ($p<0.005$).

These results indicated that adoptive transfer of CD3-AT cells itself was effective in eliminating the dissemination of tumor in the peritoneal cavity, and no further enhancement was observed, even when exogenous IL-2 was administered simultaneously. Thus, IL-2 administration was not performed in the following experiments.

To elucidate the specificity of the treatment, we

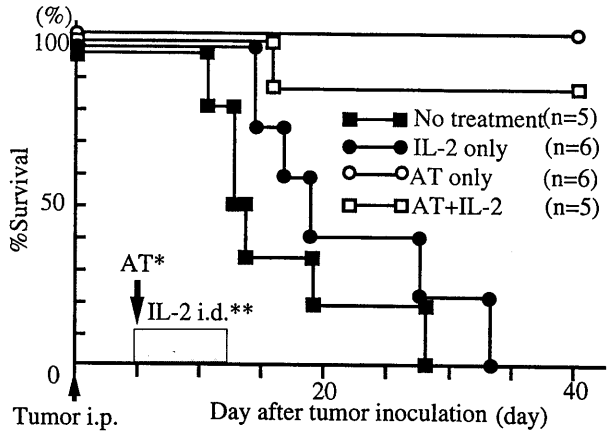


Fig. 1. Adoptive immunotherapy in mice with peritoneal MCA205 dissemination.
B6 mice were injected intraperitoneally (i. p.) with 2×10^5 MCA205 tumor cells on day 0 to initiate peritoneal dissemination. Five days later, CD3-AT cells were adoptively transferred (AT) i. p. to treatment groups. *CD3-AT cells were generated from MCA205 TDLN. **IL-2 (16000 u) was given intradermally (i. d.) once daily for 5 days. Treatment with CD3-AT cells with or without IL-2 administration resulted in a significant prolongation of survival time compared to groups with no treatment or exogenous IL-2 administration ($p<0.005$., generalized Wilcoxon test).

performed a criss-cross survival experiment using MCA 205 and 203, which are known to have distinct tumor-associated antigens. As shown in Fig. 2, while treatment with LAK cells had no effect on this peritoneal tumor dissemination model, mice receiving treatment with corresponding effector cells showed improvement. These data indicated that the antitumor effect of CD3-AT cells is clearly tumor specific.

Effect of host macrophages depletion on antitumor effect

We previously demonstrated that CD3-AT cells have potent *in vivo* antitumor activity despite minimal *in vitro* cytotoxicity. This paradoxical phenomenon could be explained if host components play an essential role in tumor regression. We assumed that there are 2 candidates for host immune cells that may participate in this anti-tumor activity: one is lymphocyte and the other macrophage. First, to investigate the involvement of the lymphocytes of the tumor-bearing host, in this antitumor activity especially of immune T cells, we carried out a survival study using the same treatment protocol with a group of 500-rad irradiated mice.

As shown in Fig. 3, pretreatment with 500 rads of irradiation had no effect on the antitumor effect. This result indicates that host T cells do not play a major role in this particular treatment model.

Since the T cells of the tumor-bearing host have no effect on the treatment, we next focused on another cell component in the peritoneal cavity, macrophages.

Using $GdCl_3$, known as Kupffer cell eliminator[12], we succeeded in depleting host peritoneal macrophages. The peritoneal cells that stained positive for F4/80 and M1/70, which are known to be expressed on the surface of macrophages, were almost completely depleted by intravenous injection of $GdCl_3$, as shown by the flow cytometric analysis in Fig. 4. This significant suppression continued for about 1 week after the administration of $GdCl_3$.

As we expected, in the macrophage depleted state, the anti-tumor effect was dramatically abrogated in the survival study, as shown in Fig. 5. This result indicates that, in contrast with host T cells, a recipient component(s), presumably macrophages, sensitive to $GdCl_3$ but relatively resistant to radiation (500 rads), is essential for the antitumor effect mediated by CD3-AT cells.

CD3-AT cells require macrophages to show *in vitro* cytotoxicity

In a previous report, we demonstrated that CD3-AT cells showed little cytotoxicity in a 4-hr ^{51}Cr releasing assay, unlike IL-2 induced non-specific LAK cells. Since previous experiments indicated that CD3-AT cells cannot mediate the antitumor effect by themselves, but instead require the participation of host components, especially macrophages, we performed *in vitro* cytotoxicity assays with coexisting peritoneal cells.

As shown in Fig. 6, CD3-AT cells showed no cytotoxic effects with or without peritoneal cells in standard 4-hr assays, while LAK cells mediated nonspecific cytotoxicity. In 18-hr assays, on the other hand, CD3-AT cells mediated tumor-specific cytotoxicity with coexisting peritoneal cells, an effect that was enhanced by the addition of M-CSF in the assay. The amount of M-CSF added in this experiment was not enough to induce the antitumor activities of M-CSF itself. A dose of M-CSF 1,000 times higher is needed to observe the antitumor effect against this mouse fibrosarcoma (data not shown).

IFN-γ plays an active role in the anti-tumor activity of CD3-AT cells

In this series of experiments, we learned that cooper-

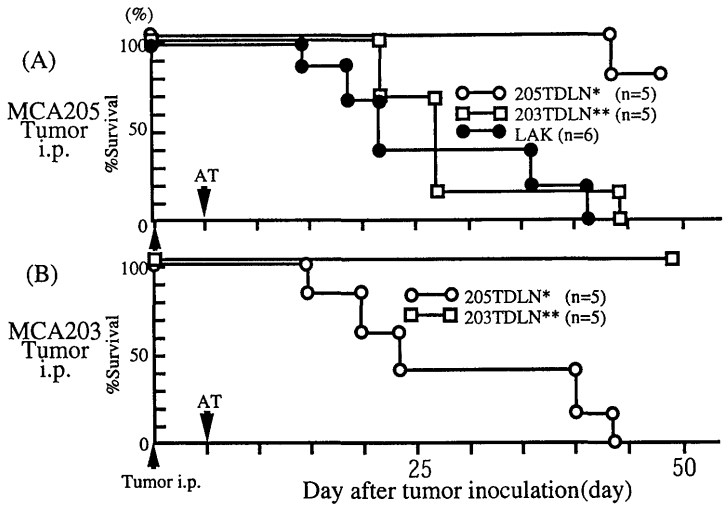
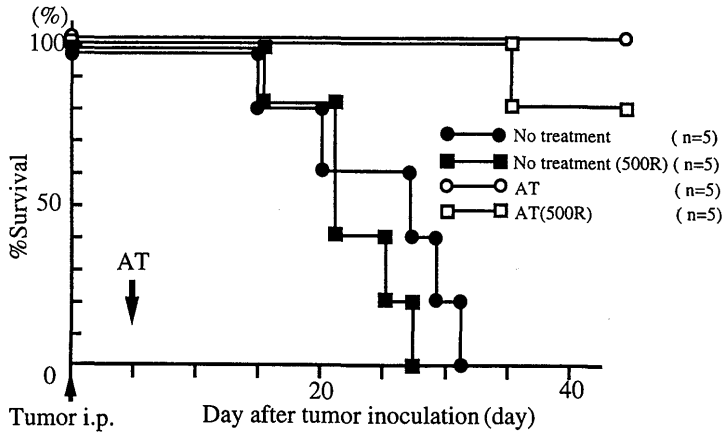


Fig. 2. Specificity of adoptive immunotherapy of mice with peritoneal tumor dissemination.
B6 mice were injected i. p. with 2×10^5 of 205 (A) and 203 (B) tumor cells on day 0 to initiate peritoneal dissemination. Five days later, CD3-AT cells from 2 different TDLN were injected i. p. in both treatment groups. LAK cells were generated by incubating normal spleen cells with 1000 U/ml of IL-2 for 3 days. *205TDLN; effector cells generated from LN draining 205. **203TDLN, effector cells generated from LN draining 203. In this criss-cross survival study, only the corresponding CD3-AT cells showed significant prolongation of survival time against their counterparts ($P<0.005$., generalized Wilcoxon test).



Flg. 3. Effect of 500-rad irradiation on adoptive immunotherapy of mice with peritoneal tumor dissemination.
A survival study was performed with the same treatment protocol as in Fig. 1. Some mice were given 500 rads whole-body irradiation 1 day prior to tumor i. p. inoculation. In the T-cell depleted state, CD3-AT cells showed comparative therapeutic effect ($p>0.1$., generalized Wilcoxon test). The results indicate that the host's T-cell component plays no major role in this treatment model.

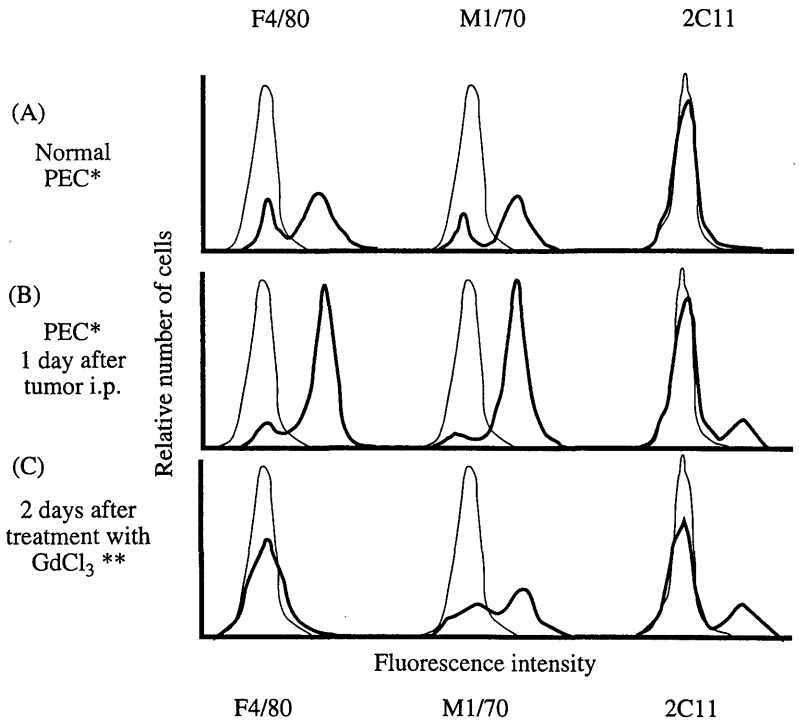


Fig. 4. Effect of macrophage depletion induced by i. v. $GdCl_3$ administration.
Flow microfluorocytometric analysis of peritoneal exudate cells was performed on the following 3 groups of mice. PEC were collected from normal mice (A), mice bearing 1-day peritoneal dissemination of 205 tumor cells (B), and mice injected with $GdCl_3$ 2 days prior to 205 tumor inoculation. *PEC, peritoneal cells were collected by washing the peritoneal cavity with 5 ml saline 4 times. **One mg of $GdCl_3$ was injected i. v. The macrophages that stained positively for F4/80 were suppressed by $GdCl_3$ administration.

ative actions occurred between adoptively transferred CD3-AT cells and host macrophages in this antitumor activity. To investigate the mediators of this activity, we performed *in vitro* culture of tumor and CD3-AT cells with and without peritoneal cells, and examined the concentration of 2 cytokines, IFN-γ and TNF-α, which are known to have antitumor and immunomodulating properties, in the culture supernatant.

As shown in Fig. 7, *in vitro* tumor cell propagation was suppressed by CD3-AT cells with coexisting peritoneal cells in the culture. Under these conditions, a significant increase in IFN-γ and TNF-α was detected by ELISA assay of the culture supernatant.

To confirm the involvement of these 2 cytokines in antitumor activity, we performed *in vivo* blocking experiments and *in vitro* cell growth assay using a monoclonal rat lgG that neutralizes the activity of murine IFN-γ, but not IFN-α or IFN-β[13], and a monoclonal rabbit lgG that neutralizes TNF-α[14]. Mice with established peritoneal dissemination were pretreated with either anti-IFN-γ or anti-TNF-α by intraperitoneal injection 1 day prior to adoptive transfer of CD3-AT cells. As shown in Fig. 8, antibody to IFN-γ significantly inhibited the *in vivo* activity of CD3-AT cells, while late administration of anti-IFN-γ after the adoptive transfer of CD3-AT cells did not. On the other hand, neither concomitant nor post-treatment anti-TNF-α antibody administration reduced the *in vivo* effect treatment. In the same manner, as shown in Fig. 9, addition of anti-IFN-γ to the *in vitro* culture inhibited susceptibility to tumor cell lysis, while anti-TNF-α did not show any effect (data not shown).

These results, which indicated that these 2 cytokines, especially IFN-γ, play an essential role in this antitumor activity, were found mostly in the early phase of the antitumor activities.

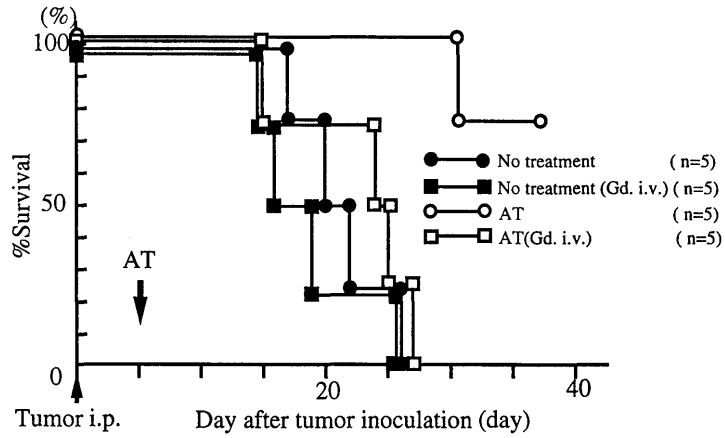
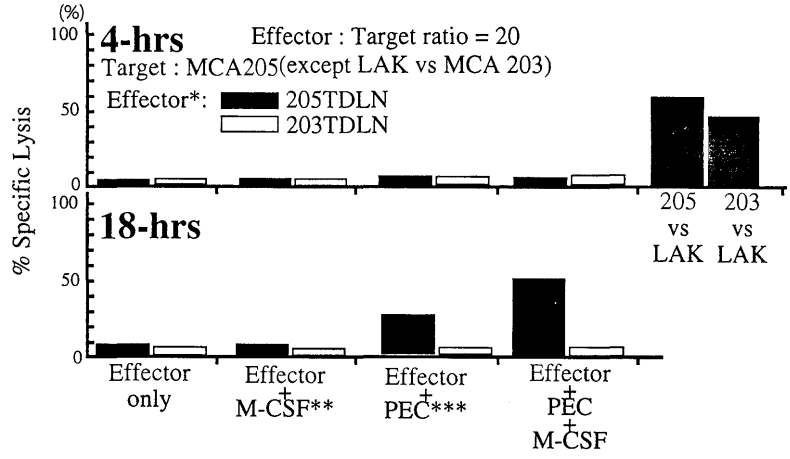


Fig. 5. Effect of macrophage depletion on adoptive immunotherapy of mice with peritoneal tumor dissemination.
A survival study was performed with the same treatment protocol as in Fig. 1.
Some mice were given 1 mg of $GdCl_3$ i. v. 1 day prior to tumor i. p. inoculation. Depletion of macrophage with $GdCl_3$ abrogated the therapeutic effect ($p<0.005$., generalized Wilcoxon test), indicating that the host's macrophage components are essential for this treatment effect.



Flg. 6. *In vitro* cytotoxicity of CD3-AT cells.
To simulate the intraperitoneal environment, PEC was added to the assay. CD3-AT cells generated from 205 or 203 TDLN were tested against ^{51}Cr-labeled 205 target cells in 4-hr or 18-hr ^{51}Cr release assays.
*Effector, Closed and open bars represent the effector cells from LN draining 205 and 203 tumors, respectively. **M-CSF, 400 U/ml added to each assigned well. ***PEC, peritoneal cells collected after 3 days of tumor i. p. inoculation. In standard 4-hr assays, CD3-AT cells showed no cytotoxic effects, although LAK cells mediated cytotoxicity, which showed no tumor specificity. In 18-hr assays, CD3-AT cells mediated tumor-specific cytotoxicity under conditions of coexistence with PEC, which was enhanced by the addition of M-CSF in the assay.

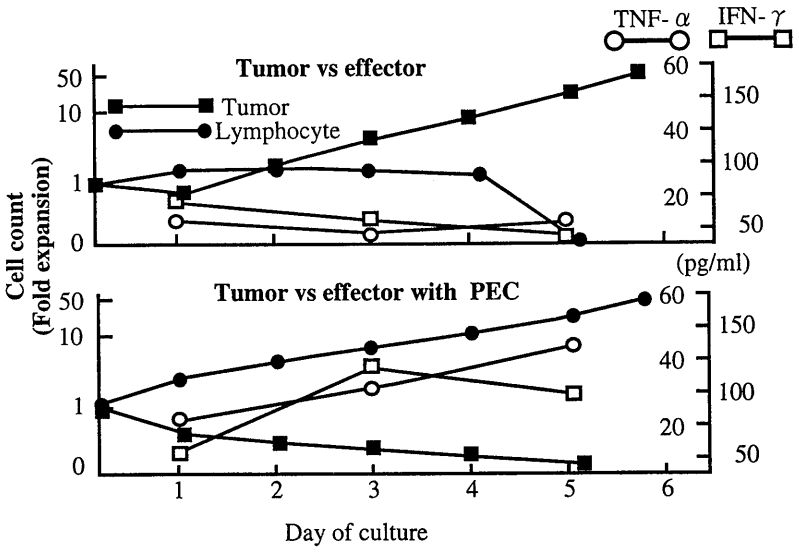
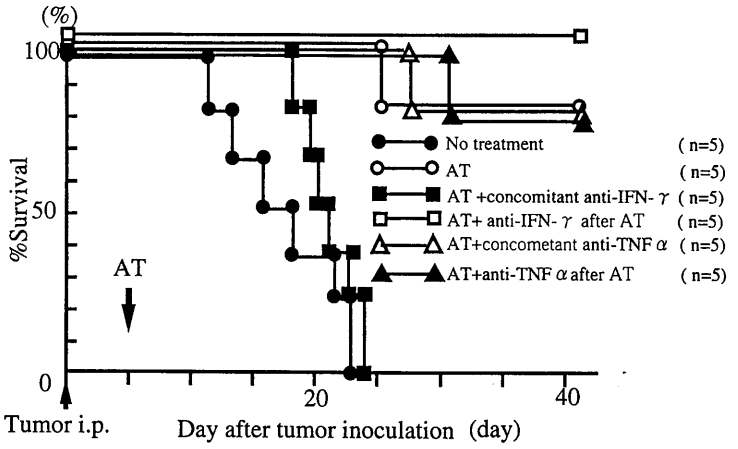
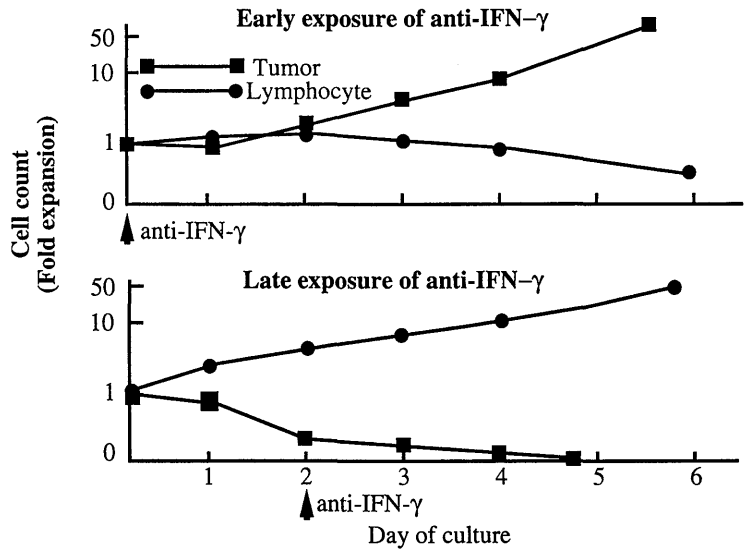


Fig. 7. Comparison of *in vitro* cell proliferation of tumor and lymphocyte 2×10^4 205 cells were admixed with 2×10^5 CD3-AT cells generated from 205 TDLN and were cultured in 2 ml complete medium. PEC were collected from mice 3 day after tumor i. p. inoculation. Four$\times10^4$ PEC were added to assigned wells.
Tumor cell propagation was suppressed by CD3-AT cells under condition of coexistence with PEC in the culture. Under these conditions, a significant increase in IFN-γ and TNF-α were detected in the culture supernatant. This indicates that these cytokines play an active roles in this anti-tumor activity.



Flg. 8. Effect of anti-IFN-γ or anti-TNF-α on adoptive immunotherapy of mice with peritoneal tumor dissemination.
A survival study was performed with the same treatment protocol as in Fig. 1. Some mice were treated with anti-IFN-γ or anti-TNF-α antibody by intraperitoneal injection concomitant to, or 2 days after, adoptive transfer of CD3-AT cells. The antitumor effect was abrogated by anti-IFN-γ administration when injected concomitant to adoptive transfer. ($p<0.005$., generalized Wilcoxon test)



Flg. 9. Effect of anti-IFN-γ mAb on *in vitro* cell growth of tumor and lymphocyte.
Two $\times 10^4$ 205 cells were cocultured with 2 $\times 10^5$ CD3-AT cells generated from 205 TDLN and 4 $\times 10^4$ PEC. Anti-IFN-γmAb was added to assigned wells on day 0 to test early exposure and on day 2 to test late exposure. Inhibition of tumor cell propagation was suppressed when anti-IFN-γ mAb was added on day 0. This indicates that IFN-γ plays an essential role in this anti-tumor activity in the early phase of tumor-effector-macrophage contact.

DISCUSSION

We have demonstrated that adoptively transferred CD3-AT cells bearing a mixed population of $CD4^+$ and $CD8^+$ T cells mediate *in vivo* antitumor activity[4]. It has been postulated that $CD4^+$ T cells mediate the *in vivo* rejection of the tumors by either eliciting a DTH response[15,16,17] or amplifying cytolytic $CD8^+$ T-cell activity[18]. Both reactions are thought to occur through soluble mediators released by the transferred T cells. Although the evidence that these processes are responsible for rejection *in vivo* is clearly indirect, it is thought that the host contributes, in some capacity, an integral component(s) to the rejection response induced by the transfer of effector cells. Host macrophages appear to be a candidate for this component in the immune response to tumors because of their direct tumoricidal activity.

As an initial attempt to elucidate some of the complex cellular interactions that lead to the regression of established syngeneic tumor, our current study was undertaken using an agent, $GdCl_3$, that is selectively toxic to macrophages. In the experiments reported in this paper, we have demonstrated that $GdCl_3$ treatment of mice with established peritoneal dissemination of MCA fibrosarcoma abrogates the curative capacity of adoptively transferred CD3-AT cells. Findings of a similar nature have been reported in the adoptive immunotherapy of a syngeneic tumor in guinea pigs[19] and syngeneic mouse lymphomas[20]. In those two studies, the investigators used trypan blue and carrageenan, which have a known, relatively selective, toxicity to macrophages. The reasons why we chose $GdCl_3$ as a macrophage depletor are, first, in this particular peritoneal dissemination model, we failed to deplete the peritoneal macrophages by carrageenan administration, causing rather inflammatory cell infiltration, and second, $GdCl_3$ administration has little effect on host cell components other than macrophages, which was confirmed in FACS analysis of peritoneal cells using the macrophage-specific antibodies F4/80[21] and M1/70[22].

According to the result of the survival study and of the *in vitro* 18-hr cytotoxicity test, in this particular peritoneal dissemination model, host macrophages, sensitive to $GdCl_3$ but relatively resistant radiation (500 R), are essential to the expression of adoptive immunity and cure of tumor-bearing mice. It appears

that definite cooperation exists between adoptive transferred effector cells and host macrophages. One possible explanation for the lack of correlation observed between *in vivo* antitumor activity and *in vitro* cytotoxicity is that adoptively transferred cells may secrete cytokines in response to tumors. The secreted cytokines may then participate in tumor regression by either direct antitumor activity or activation and recruitment of host effector cells and macrophages. We chose 2 cytokines, IFN-γ and TNF-α, since we have detected an increase of these 2 cytokines in culture supernatant and because they have been reported to possess both direct antitumor activity[23,24] and some immunomodulating properties, including: 1) increasing MHC antigen expression on tumor cells[25], 2) macrophage activation[26], 3) activation of cytotoxic T-lymphocytes[27], 4) enhancing the susceptibility of tumor cells to lysis[28], and 5) mediation of delayed-type hypersensitivity[29].

Interestingly, our results demonstrate that the increase in secretion of these two cytokines occurs under conditions of coexisting host macrophages, which indicates that there is some kind of feedback regulation of cytokine release, not only with tumor-effector relations, but also with the indispensable participation of host macrophages, which are activated or primed by the cytokines.

In vivo and *in vitro* antibody blocking experiments have revealed the essential role of IFN-γ. Since antibody blocking inhibited treatment efficacy only before and not after the adoptive transfer, IFN-γ is needed most in the early phase of the tumor-effector-macrophage contact, maybe through the first three properties out of the five previously mentioned, first, MHC expression enhancement of tumor cells, second, macrophage activation, and third, cytotoxic T-cell activation. Tuttle et al. also reported that IFN-γ is the key cytokine in adoptive immunotherapy of mice sarcoma because of the antiproliferative effect of the cytokine itself and the activation of host effector cells[30]. In our study, however, host macrophages were shown to be essential for tumor eradication, and IFN-γ is more important for its macrophage activation than for its direct antiproliferative effect on the tumor.

Presumably, macrophages are not only passively primed and activated by cytokines, but also actively regulate cytokine release at the tumor site, and TNF-α could be the one of the mediators. Although we detected increased TNF-α in culture supernatant, antibody blocking against TNF-α did not abrogate the treatment effect like anti-IFN-γ. However, the dose and timing of antibody administration may not have been adequate block TNF-α. The exact role of TNF-α, if any, is not clear from our study but, we assumed that the activated macrophages may release TNF-α, which may be synergistic with IFN-γ and contribute to the total therapeutic effect against tumor cells.

This report presents the important idea that adoptive immunotherapy with CD3-AT cells, which have potent *in vivo* antitumor activity despite minimal *in vitro* cytotoxicity, require host components, mainly macrophages, participating at the tumor eradication site and being mediated by IFN-γ and TNF-α.

In this particular peritoneal dissemination model, effector-host cell collaboration might be different from systemic i. v. administration of effector cells, but we found that this model is a rather interesting way of studying the anti-tumor mechanisms of adoptive immunotherapy, and may be applicable to human cancer patients not only in systemic therapy, but also for controlling local disease, such as malignant peritonitis or pleuritis.

Furthermore, these data suggest that it may be possible to enhance therapeutic efficacy by using other macrophage activators such as M-CSF or GM-CSF, in the clinical application of adoptive immunotherapy, and that gene therapy with IFN-γ or TNF-α transfected effector cells may improve treatment efficacy in human cancer patients.

Acknowledgments. This work was supported in part by a Niigata prefecture research grant, a Niigata University Research Grant, and a Tsukada Memorial Research Grant.

REFERENCES

1) Rosenberg SA, Terry WD: Passive immunotherapy of cancer in animals and man. *Adv Cancer Res* **25:** 323, 1977.
2) Fernandez-Cruz E, Halliburton EB, Feldman JD: In vivo elimination by specific effector cells of an established syngeneic rat moloney virus-induced sarcoma. *J Immunol* **123:** 1772, 1979.
3) Shu S, Rosenberg SA: Adoptive immunotherapy of a newly induced sarcoma: immunologic characteristics of effector cells. *J Immunol* **135:** 2895, 1985.
4) Yoshizawa H, Sakai K, Chang AE, Shu SY: Activation by anti-CD3 of tumor-draining lymph node cells for specific adoptive immunotherapy. *Cell Immunol* **134:** 473-479, 1991.
5) Yoshizawa H, Chang AE, Shu S: Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. *J Immunol* **147:** 729-737, 1991.
6) Yoshizawa H, Chang AE, Shu S: Cellular interac-

tions in effector cell generation and tumor regression mediated by anti-CD3/interleukin 2-activated tumor-draining lymph node cells. *Cancer Res* **52:** 1129-1136, 1992.

7) Mitsuma S, Yoshizawa H, Ito K, Moriyama H, Wakabayashi M, Chou T, Arakawa M, Shu S: Adoptive immunotherapy mediated by anti-TCR/IL-2-activated tumour-draining lymph node cells. *Immunology* **83:** 45-51, 1994.

8) Miyao H, Chou T, Ito K, Moriyama H, Mitsuma S, Wakabayashi M, Yosizawa H, Arakawa M: Treatment of advanced primary lung cancer associated with malignant pleural effusion by the combination of immunotherapy and chemotherapy. *Oncology* **51:** 87-94, 1994.

9) Ito K, Chou T, Mitsuma S, Moriyama H, Wakabayashi M, Mizusawa A, Miyao H, Yoshizawa H, Tsuchiya T, Ebe T, Yuejing L, Arakawa M: A novel chemo-immunotherapy for advanced non-small cell lung cancer. *J Jpn Soc Cancer Ther* **29:** 36-46, 1993.

10) Barth RJ, Bock SN, Mule JJ, Rosenberg SA: Unique murine tumor-associated antigens identified by tumor-infiltrating lymphocytes. *J Immunol* **144:** 1531, 1990.

11) Rosenberg SA, Grimm EA, McGrogan M, Doyle M, Kawasaki E, Koths K, Mark DF: Biological activity of recombinant hunman interleukin-2 produced in E. coli. *Science* **223:** 1412, 1984.

12) Hardonk MJ, Dijkhuis FWJ, Hulstaert CE, Koudstaal J: Heterogeneity of rat liver and spleen macrophages in gadolinium chloride-induced elimination and repopulation. *J Leukocyte Biol* **52:** 751-763, 1992.

13) Spitalny GL, Havell EA; Monoclonal antibody to murine γ interferon inhibits lymphokine-induced antiviral and macrophage tumorcidal activities. J *Exp Med* **159:** 1560-1595, 1984.

14) Franks AK, Kujawa KI, Yaffe LJ: Experimental elimination of tumor necrosis factor in low-dose endotoxin models has variable effects on survival. Infect. Immunity, **59:** 2609-2614, 1991.

15) Kim B, Rosenstein M, Weiland D, Eberlein TJ, Rosenberg SA: Clonal analysis of the lymphoid cells mediating skin allograft rejection. *Transplantation* (Baltimore) **36:** 525-532, 1983.

16) Leclerc JC, Cantor HT: T cell-mediated immunity to oncoRNA virus-induced tumors. *J Immunol* **124:** 851-854, 1980.

17) Loveland BE, Hogarth PM, Ceredig RH, McKenzie IFC: Cells mediating graft rejection in the mouse. I. Lyt-1 cells mediate skin graft rejection. *J Exp Med* **153:**1044-1057, 1981.

18) Millis CD, North RJ: Expression of passively transferred immunity against an established tumor depends on generation of cytolytic T cells in recipients. *J Exp Med* **157:** 1448-1460, 1983.

19) Shu S, Fonseca LS, Kato H, Zbar B: Mechanisms of immunological eradication of syngeneic guinea pig tumor; participation of component(s) of recipient origin in the expression of systemic adoptive immunity. *Cancer Res* **43:** 2637-2643, 1983.

20) Mule JJ, Rosenstein M, Shu S, Rosenberg SA: Eradication of a disseminated syngeneic mouse lymphoma by systemic adoptive transfer of immune lymphocytes and its dependence upon a host component(s). *Cancer Res* **45:** 526-531, 1985.

21) Hume DA, Robinson AP, Macpherson GG, Gordon S: The mononuclear phagocyte system of the mouse defined by immunohistochemical localization of antigen F4/80. *J Exp Med* **158:** 1522-1536, 1983.

22) Springer T: Monoclonal antibody analysis of complex biological systems; combination of cell hybridization and immunoadsorbents in a novel cascade procedure and its application to the macrophage cell surface. *J Biol Chem* **256:** 3833-3839,1981.

23) Ratliff TL, Kadmon D, Shapiro A, Jacobs AJ, Heston WDW: Inhibition of mouse bladder tumor proliferation by murine interferon-γ and its synergism with interferon-b. *Cancer Res* **44:** 4377-4381, 1984.

24) ASher AL, Mule JJ, Rosenberg SA: Recombinant human necrosis factor mediates regression of a murine sarcoma in vivo via Lyt-2+cells. *Cancer Immunol Immunother* **28:** 153-156, 1989.

25) De Jong R, Stokkers P, Lamme E, Kool JM, Borst F, Brouwer, M, Miedema F, Van Lier RAW: Regulation of T-cell differentiation by CD2 and CD28 accessory molecules. *Immunology* **74:** 175-182,1991.

26) Pace JL, Russel SW, Torres BA, Johnson HM, Gray PW: Recombinant mouse γ interferon induces the prime step in macrophage activation for tumor cell killing. *J Immunol* **130:** 2011-2013, 1983.

27) Shimizu Y, Iwatsuki S, Herberman RB, Whiteside TL: Effects of cytokines on in vivo growth tumor-infiltrating lymphocytes obtained from human primary and metastatic liver tumors. *Cancer Immunol Immunother* **32:** 280-288, 1991.

28) Fox BA, Spiess PJ, Kasid A, Puri R, Mule JJ, Weber JS, Rosenberg SA: In vitro and in vivo antitumor properties of a T-cell clone generated from murine tumor-infiltrating lymphocytes. *J Biol Response Modif* **9:** 499-511, 1990.

29) Fong TA, Mosman TR: The role of IFN-γ in delayed type hypersensitivity mediated by Th1 clones. *J Immunol* **143:** 2887-2893, 1989.

30) Tuttle TM, McCrady CW, Inge TH, Salour M, Bear HD: γ-Interferon plays a key role in T-cell-induced tumor regression. *Cancer Res* **53:** 833-839, 1993.