Cancer Immunol Immunother (1995) 40: 173–181

© Springer-Verlag 1995

ORIGINAL ARTICLE

Tadao Okamoto · Mamoru Harada · Yoshihiro Shinomiya
Goro Matsuzaki · Kikuo Nomoto

The antitumor effect of tumor-draining lymph node cells activated by both anti-CD3 monoclonal antibody and activated B cells as costimulatory-signal-providing cells

Received: 2 August 1994 / Accepted: 16 November 1994

Abstract To establish an efficient cell-culture system for adoptive immunotherapy, we attempted to use lipopolysaccharide(LPS)-activated B cells (LPS blasts) as costimulatory-signal-providing cells in the in vitro induction of antitumor effector cells. Both normal and tumor-draining lymph node cells were efficiently activated by both anti-CD3 monoclonal antibody (mAb) and LPS blasts, and subsequently expanded by a low dose of interleukin-2 (IL-2; anti-CD3 mAb and LPS blasts/IL-2). The expanded cells were predominantly $CD8^+$ T cells and showed a low level of tumor-specific cytotoxic T lymphocyte (CTL) activity. The adoptive transfer of B16-melanoma-draining lymph node cells expanded by anti-CD3 mAb and LPS blasts/IL-2 showed significant antitumor effect against the established metastases of B16 in combination with intraperitoneal injections of IL-2. This treatment cured all B16-bearing mice. In addition, these mice also showed tumor-specific protective immunity against B16 at the rechallenge. Considering that activated B cells express several kinds of costimulatory molecules, these findings thus indicate an efficacy of costimulation that is derived from activated B cells for the in vitro induction of tumor-specific CTL, in co-operation with anti-CD3 mAb. The culture system presented here may thus be therapeutically useful, providing potent effectors for adoptive immunotherapy against various types of cancer.

Key words Adoptive immunotherapy · Costimulation · Activated B cells · Anti-CD3 mAb

T. Okamoto · Y. Shinomiya · G. Matsuzaki · K. Nomoto
Department of Immunology, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812, Japan

M. Harada (✉)
Department of Virology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812, Japan

Introduction

Almost all tumor-bearing hosts are unable to reject tumors even though the tumors are immunogenic, and, therefore, tumor-specific cytotoxic T lymphocyte (CTL) precursors are prepared in the hosts [6, 10]. To explain this phenomenon, several researchers have reported that the tumor-bearing state is accompanied by several kinds of immunosuppression [18, 19, 28]. Therefore, in order to avoid in vivo immunosuppression, in vitro culture systems have been proposed to prepare antitumor T cells for adoptive immunotherapy [25]. Cultured effector T cells have been able to show antitumor activity in some murine models [26] and therapeutic efficacy has also been reported in the clinical field [20, 22].

In establishing a successful culture system to expand tumor-specific T cells, there are several difficulties, the most significant being that we can not always obtain a sufficient number of autologous tumor cells for the in vitro restimulation. Despite this, Yoshizawa et al. have described a practically useful culture system for clinical application [30], reporting that the transfer of tumor-draining lymph node cells cultured with anti-CD3 monoclonal antibody (mAb) and subsequently with interleukin-2 (IL-2) was able to show antitumor specific activity against established pulmonary metastases. However, in their culture system, the degree of expansion of effector cells was not very large (only severalfold). Since the number of effector cells to be transferred is critical for the therapeutic effect of adoptive immunotherapy [10], we suppose that it would be therapeutically useful to improve the culture system by which a sufficient number of effector cells could be obtained from limited cell sources.

Recent reports have revealed that T cells need a costimulatory signal (a second signal) other than the signal through the T cell receptor (the first signal) for their optimal activation [23]. The first signal without the costimulatory signal results in T cell inactivation [24]. The costimulatory signal through CD28 molecules has been definitely characterized [7, 12], while dendritic cells, macrophages and

activated B cells are reported to express the counterparts of CD28 molecules such as B7 [2, 8, 14].

In the present study, we tried to utilize activated B cells as costimulatory-signal-providing cells for the in vitro induction of antitumor effector T cells. We demonstrated that tumor-draining lymph node cells, which had been cultured with anti-CD3 mAb and LPS blasts and subsequently with a low dose of IL-2, showed antitumor activity against the established metastatic melanoma. The mice surviving this treatment showed tumor-specific protective immunity against melanoma at the rechallenge. The efficacy of activated B cells for the in vitro induction of antitumor T cells is also discussed.

Materials and methods

Mice

Female C57BL/6 (B6) mice, 6–7 weeks of age, were obtained from Japan SLC Inc. (Hamamatsu, Shizuoka, Japan). All mice were bred in specific-pathogen-free conditions. They were later used for experiments at 8 weeks of age.

Tumors

B16 is a melanoma of B6 origin. Both MCA, 3-methylcholanthrene-induced fibrosarcoma, and EL-4, T cell lymphoma, are also of B6 origin. All tumor cell lines were maintained in vitro.

IL-2

Highly purified human recombinant IL-2 was kindly provided by Takeda Chemical Industries, Ltd., Osaka, Japan. The specific activity of IL-2 was 1.4×10^7 Japan reference units/mg protein. When the Biological Response Modifiers Program standard was used, it corresponded to 1.2×10^7 units/mg protein. In this report, the unit of IL-2 is Japanese reference units.

Anti-CD3 mAb

Anti-CD3 mAb was purified from the supernatants of anti-CD3-mAb-producing hybridoma 145-2C11 (kindly provided by Dr. J. A. Bluestone, Department of Pathology, University of Chicago, Chicago, Ill.). The antibody was obtained by collecting supernatants of the hybridoma cells growing in a serum-free medium (101; Nissui Pharmaceutical Co. Ltd., Tokyo, Japan).

Preparation of LPS blasts

RPMI-1640 medium (Gibco, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum (HyClone, Longan, Utah), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin, 10 mM HEPES and 0.02% sodium bicarbonate was used as the complete culture medium. Spleen cell suspensions (4×10^6 cells/ml) obtained from untreated B6 mice were cultured with 5 μg/ml lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) for 2 days in the complete culture medium. LPS blasts were enriched by Percoll (Sigma Chemical Co., St. Louis, Mo.) gradient centrifugation. The Percoll gradient was prepared by laying 40% Percoll containing LPS-stimulated spleen cells over 50% Percoll, and centrifuged at 3000 rpm for 13 min at 4 °C. The cells at the interface were collected and washed three times with the complete medium. The enriched cells were $94 \pm 3\%$ $B220^+$ and less than 1% $CD3^+$ (data not shown).

Tumor-draining lymph node cells

B6 mice were subcutaneously (s.c.) inoculated in the abdomen with 1×10^6 B16 or MCA bilaterally and the draining lymph nodes were harvested on day 12 or 15. The lymph nodes from the untreated B6 mice were used as the control.

In vitro culture with anti-CD3 mAb and/or LPS blasts and subsequently with IL-2

Either normal or tumor-draining lymph node cells (1×10^6 cells/ml) were cultured with anti-CD3 mAb (1 μg/ml) and/or LPS blasts (3×10^6 cells/ml) in six-well plates (Costar 3516, Costar, Cambridge, Mass.) at 37 °C in 5% CO_2 for 1 or 2 days. The cultured cells were harvested and the viable lymphocytes were enriched by using Lympholyte-M (Cedarlane Laboratories, Ontario, Canada). Thereafter, the viable cells were cultured at a cell dose of $(2-4) \times 10^5$ cells/ml in six-well plates or in a 225-cm² tissue-culture flask (Costar 3001, Costar) in the complete culture medium containing 20 U/ml or 50 U/ml IL-2 for the indicated length of time. These cultured lymph node cells were then used for adoptive immunotherapy.

Flow-cytometric analysis

For flow cytometry, a cell suspension was incubated with the following mAb and analyzed by FACScan (Becton Dickinson, Mountain View, Calif.). Phycoerythrin(PE)-conjugated anti-(mouse CD4) mAb, PE-conjugated anti-(mouse CD8) mAb, PE-conjugated-Streptavidin, and fluorescein-isothiocyanate-conjugated-Streptavidin were purchased from Gibco BRL (Gaithersburg, Md.). To exclude any dead cells, the prepared cells were stained with 2 μg/ml propidium iodide (Sigma Chemical Co.).

Proliferation assay

The proliferation of the cells was assayed by standard [^{3}H]dThd incorporation and scintillation counting. T cells from normal lymph node cells were purified by nylon-wool passage. The percentage of $CD3^+$ T cells in purified cells was 95% (data not shown). Prepared LPS blasts were treated with 1% paraformaldehyde (Wako Pure Chemical Industries, Osaka, Japan) for 5 min and washed twice by phosphate-buffered solution and two times by the complete medium. Samples containing 2×10^5 T cells and/or fixed LPS blasts with or without anti-CD3 mAb (1 μg/ml) were seeded in each well of flat-bottomed 96-well culture plates (Coster 3595, Coster Corp., Cambridge, Mass.) in a total volume of 0.2 ml and cultured for 48 h. The cells were then harvested and the amount of incorporated [^{3}H]dThd was counted with a Beta Plate system (Pharmacia LKB Biotechnology, Uppsala, Sweden). To examine the participation of Fc receptor (FcR) in T cell proliferation, the supernatant of either anti-FcRγII/III-mAb-producing hybridoma (2.4G2: rat IgG2b) or anti-Mac-1α-mAb-producing hybridoma (M1/70.15.11.5: rat IgG2b) was added to each well.

Assay of cytotoxicity

We determined whether or not antitumor-specific CTL could be detected in the in vitro expanded tumor-draining lymph node cells. The in vitro cytolytic activity of the effector cells was examined in a standard 4-h ^{51}Cr-release assay, as previously reported [13]. The specific release was calculated according to the following formula:

$$\text{Specific } ^{51}\text{Cr release (\%)} = \frac{\text{test release (cpm)} - \text{spontaneous release (cpm)}}{\text{total release (cpm)} - \text{spontaneous release (cpm)}} \times 100$$

Spontaneous release was determined by the supernatant of the sample that was incubated with no effector cells, and the total release was determined by the supernatant of the sample, that was incubated with 10% Triton X (Wako Pure Chemical Industries).

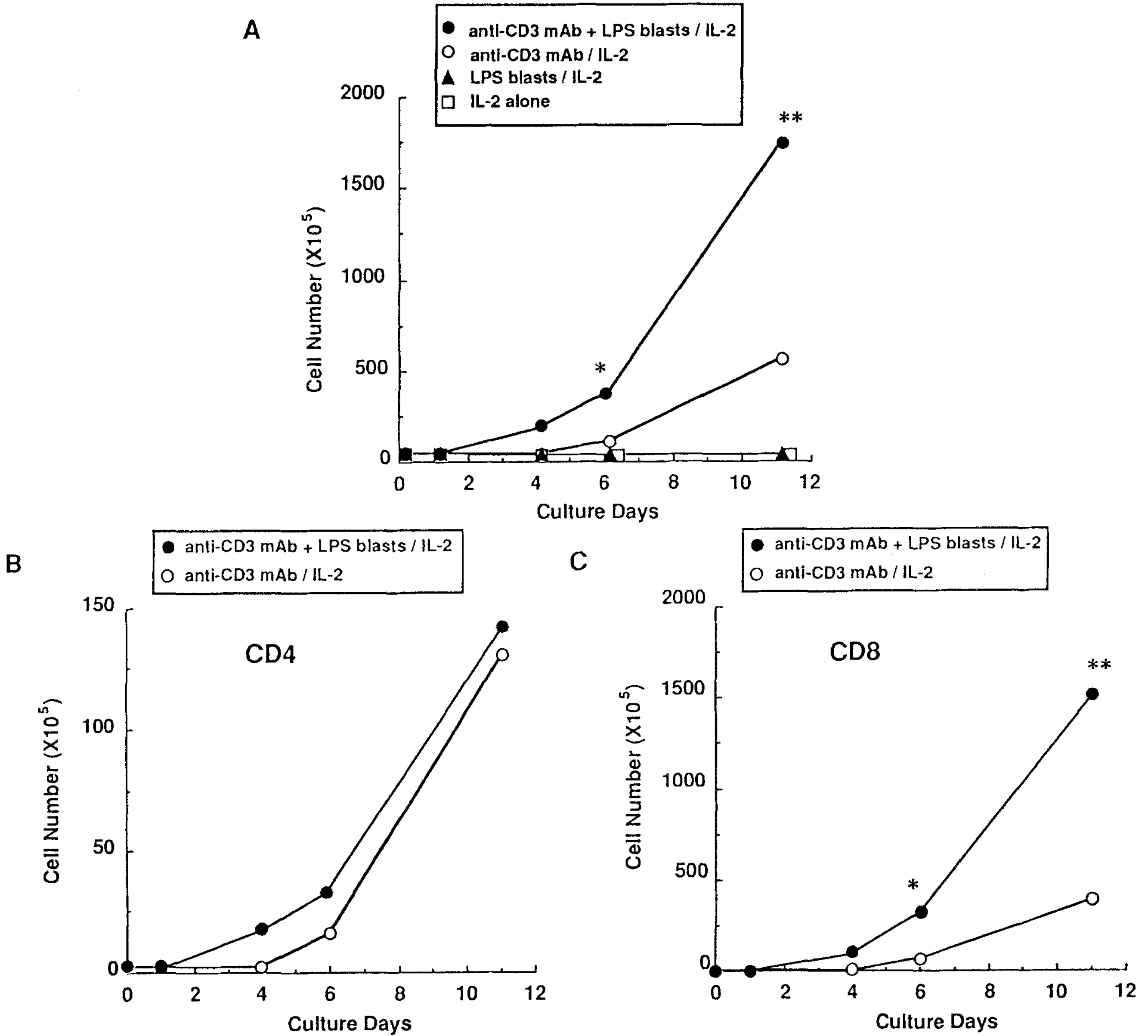


Fig. 1A–C The efficient expansion of naive lymph node cells in the culture with anti-CD3 mAb and lipopolysaccharide (*LPS*) blasts/interleukin-2 (*IL-2*). **A** Naive lymph node cells (1×10^6 cells/ml) were cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (3×10^6 cells/ml) (●), anti-CD3 mAb alone (○) or LPS blasts alone (▲) for 1 day, and subsequently with 20 U/ml IL-2 for 10 days in six-well plates. The naive lymph node cells were cultured with 20 U/ml IL-2 alone (□) throughout the culture. The numbers of $CD4^+$ (**B**) and $CD8^+$ (**C**) T cells were also determined. The number of $CD4^+$ or $CD8^+$ T cells were calculated by multiplying the total number of cultured cells by the positive percentage of $CD4^+$ or $CD8^+$ T cells. The percentage was then determined by flow cytometry. The values represent the averages of triplicate cultures that varied by no more than 10%. The same results were obtained in five similar experiments. * $P < 0.05$ compared with the other groups; ** $P < 0.01$ compared with the other groups

Adoptive immunotherapy model

To establish a model of metastasis, B6 mice were intravenously (i. v.) injected with 1×10^5 B16 melanoma cells at a volume of 0.5 ml through the tail vein. After 3 days, when micrometastases had become established on the lungs [30, 31], they were transferred i. v. with the cultured cells. Then on day 21 or 28, B6 mice were sacrificed so that the numbers of the pulmonary colonies could be counted. On day 3, some mice were intraperitoneally (i. p.) injected with 30000 U IL-2 at a volume of 0.5 ml twice daily for 4 days. Any metastatic colonies too numerous to count were assigned the arbitrary value of 350 because this was the largest number of tumor nodules that could be reliably enumerated.

Statistics

The statistical significance of the data was determined by the Mann-Whitney *U*-test. A *P* value of less than 0.05 was considered to be statistically significant.

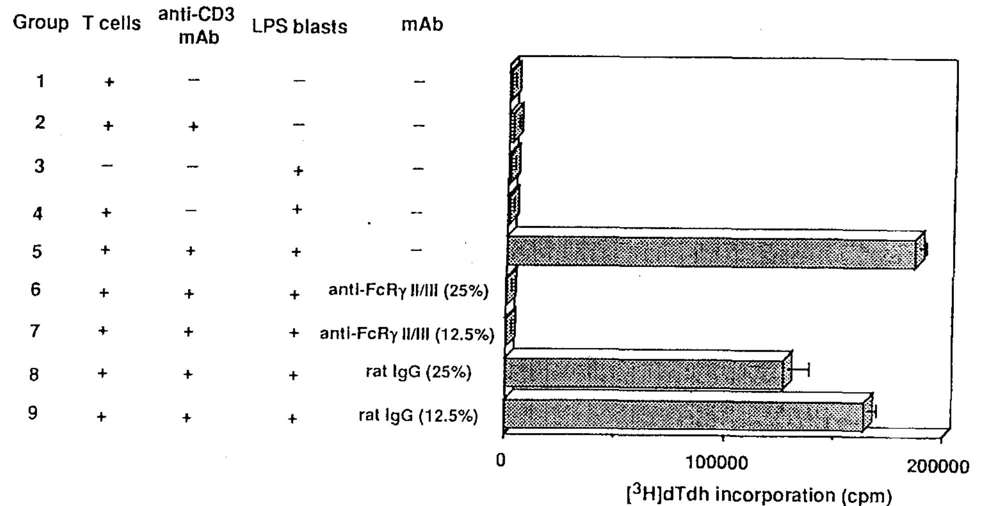


Fig. 2 Participation of Fc receptor (*FcR*) on activated B cells in the optimal activation of T cells in the presence of anti-CD3 mAb and LPS blasts. T cells (2×10^5), enriched from lymph nodes of normal B6 mice, were cultured with or without soluble anti-CD3 mAb (1 μg/ml) and/or fixed LPS blasts (2×10^5) for 48 h. The supernatant of either anti-FcRγII/III mAb (rat IgG2b) or control anti-Mac-1α mAb (rat IgG2b) was added to each well at a dose of 25% or 12.5% at the initiation of the culture. [^{3}H]dThd was added to the cultures 6 h before the harvest. The data were expressed as the mean radioactivity (cpm) ± SD of three samples

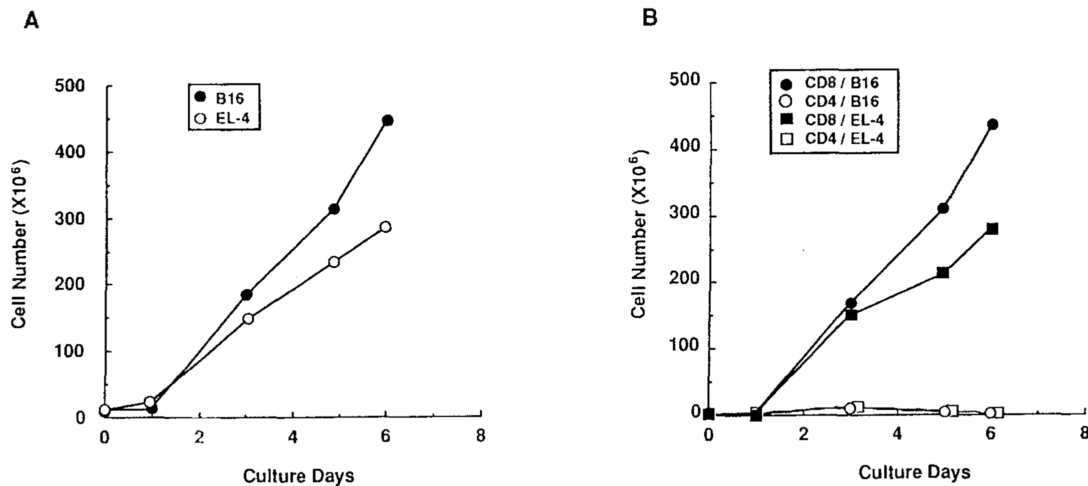


Fig. 3A, B The efficient expansion of tumor-draining lymph node cells in the culture with anti-CD3 mAb and LPS blasts/IL-2. Tumor-draining lymph node cells (1×10^6 cells/ml), from B6 mice bearing B16 or EL-4 for 12 days, were cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (3×10^6 cells/ml) for 1 day, and subsequently with 20 U/ml IL-2 for 5 days in six-well plates. On the indicated days, the cultured cells were harvested and the phenotypes of the cultured cells were analyzed by flow cytometry, using the same method shown in Fig. 1. The values represent the averages of triplicate cultures that varied by no more than 10%. The same results were obtained in three similar experiments. In Fig. 3B, on day 6, significant differences were observed between the CD8/B16 and the CD4/B16 ($P < 0.01$), and between the CD8/EL-4 and the CD4/EL-4 ($P < 0.01$)

Results

The efficient proliferation of normal lymph node cells in the culture with both anti-CD3 mAb and LPS blasts and subsequently a low dose of IL-2

It has been reported that activated B cells express several kinds of costimulatory molecules [5, 9, 15, 16]. In addition, it has also been reported that the proliferation of T cells in the presence of anti-CD3 mAb and activated B cells is due to the delivery of costimulatory signals from activated B cells [15, 17]. We have also confirmed that the optimal proliferation of T cells in the presence of anti-CD3 mAb and LPS blasts is inhibited by the addition of CTLA4 Ig, which is known to bind to B7-1 or B70 (submitted for publication). These lines of evidence led us to use activated B cells as costimulatory-signal-providing cells in the in vitro induction of antitumor effector T cells. First, we determined whether or not the additional presence of LPS blasts could enhance the cell growth of normal lymph node cells in cooperation with anti-CD3 mAb and subsequent IL-2. Figure 1A shows that the lymph node cells did not proliferate in the culture with either 20 U/ml IL-2 alone or LPS blasts and a subsequent 20-U/ml dose of IL-2 (LPS blasts/IL-2). On the other hand, culture with anti-CD3 mAb and a subsequent dose of 20 U/ml IL-2 (anti-CD3 mAb/IL-2) could induce a moderate level of cell growth. As expected, the lymph node cells were able to proliferate more efficiently in the culture with anti-CD3 mAb, LPS blasts and a subsequent dose of 20 U/ml IL-2 (anti-CD3 mAb and LPS blasts/IL-2). After 11 days of culture, the

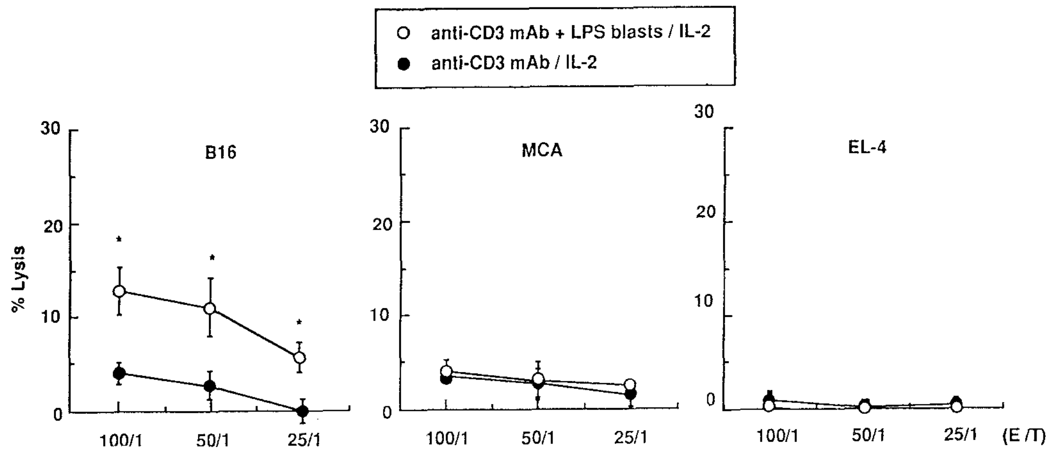


Fig. 4 Antitumor-specific cytotoxic T lymphocyte activity of tumor-draining lymph node cells expanded by the culture with anti-CD3 mAb and lypopolysaccharide (*LPS*) blasts/IL-2. Tumor-draining lymph node cells (1×10^6 cells/ml) from B6 mice bearing B16 melanoma for 15 days were cultured with anti-CD3 mAb (1 µg/ml) with or without LPS blasts (3×10^6 cells/ml) for 2 days, and then cultured with 50 U/ml IL-2 for 5 days. These cultured cells were analyzed for their cytolytic activities (4 h) against three tumor cell lines. * $P < 0.01$ significant compared with the other group

cell growth of the lymph node cells in the culture with anti-CD3 mAb and LPS blasts/IL-2 was three times as efficient as that in the culture with anti-CD3 mAb/IL-2.

We also examined the phenotype of the T cells that expanded in the culture with either anti-CD3 mAb/IL-2 or anti-CD3 mAb and LPS blasts/IL-2. Figure 1B shows that the degree of expansion of $CD4^+$ T cells was low and not different in either system. On the other hand, the degree of expansion of $CD8^+$ T cells was larger in the culture with anti-CD3 mAb and LPS blasts/IL-2 than with anti-CD3 mAb/IL-2 (Fig. 1C). These results thus suggest that the cocultures with anti-CD3 mAb and LPS blasts were preferentially capable of activating the $CD8^+$ T cells.

We next examined the participation of FcR in the optimal proliferation of T cells in the presence with both anti-CD3 mAb and LPS blasts. We determined whether or not the proliferation of enriched T cells in the presence of both anti-CD3 mAb and LPS blasts could be inhibited by the addition of anti-FcRγII/III mAb. Figure 2 shows that the addition of anti-FcRγII/III mAb completely inhibited the proliferation of T cells. In contrast, the addition of anti-Mac-1α mAb, as an isotype-matched control mAb, showed a marginal level of inhibition. These findings indicate that the FcR on LPS blasts participates in the optimal proliferation of T cells in the presence of both anti-CD3 mAb and LPS blasts.

The efficient proliferation and tumor-specific CTL activity of tumor-draining lymph node cells in the culture with anti-CD3 mAb and LPS-blasts/IL-2

We next determined whether tumor-draining lymph node cells could expand or not in the culture with anti-CD3 mAb and LPS blasts/IL-2. Figure 3A shows that the tumor-draining lymph node cells from B16- or EL-4-bearing B6 mice were able to expand in the culture with anti-CD3 mAb and LPS blasts/IL-2. A flow-cytometric analysis of the phenotype of the expanded T cells showed that $CD8^+$ T cells expanded exclusively (Fig. 3B). These results indicate that the culture with anti-CD3 mAb and LPS blasts/IL-2 resulted in a predominant expansion of the $CD8^+$ T cells in the tumor-draining lymph node cells.

We also examined the CTL activity of the B16-melanoma-draining lymph node cells, which were expanded in the culture of anti-CD3 mAb with or without LPS blasts and followed by a low dose of IL-2. Figure 4 shows that the B16-melanoma-draining lymph node cells expanded in the culture with both anti-CD3 mAb and LPS blasts and, when this was followed by a low dose of IL-2, showed a low but significant level of B16-specific CTL activity.

The antitumor effect of the tumor-draining lymph node cells expanded in the culture with anti-CD3 mAb and LPS blasts/IL-2

Up to now, we have demonstrated that the tumor-draining lymph node cells were able to expand in the culture with anti-CD3 mAb and LPS blasts/IL-2 and that such cells could show tumor-specific CTL activity in vitro. Next, we investigated whether or not the antitumor activity of the tumor-bearing lymph node cells, which expanded in this culture system, could show any antitumor activity against the established metastatic B16 melanoma. Tumor-draining lymph node cells from B6 mice bearing B16 for 15 days were cultured with anti-CD3 mAb and LPS blasts for 2 days and subsequently with 50 U/ml IL-2 for 3 or 5 days. As shown in Table 1, the i.p. injections of IL-2 alone (group 2) or the transfer of cultured cells alone (group 3), showed moderate levels of antitumor activity. The transfer of lymphokine-activated killer cells in combination with IL-2 also showed a moderate antitumor effect (group 5). On the other hand, antitumor activity was significantly elicited by the transfer of cultured cells and subsequent i.p. injections of IL-2 (group 4). We also examined the antitumor activity of the cultured cells with anti-CD3 mAb

Table 1 The antitumor effect of the B16-draining lymph node cells expanded in culture with anti-CD3 mAb and lipopolysaccharide (*LPS*) blasts/interleukin-2 (*IL-2*)

Expt.	Effector cells[a]				IL-2[b] injected (U)	Mean number of pulmonary metastases ± SEM[c]
	Group	Cultured with anti-CD3 mAb and LPS blasts	Cultured with IL-2	Number of cells transferred		
1	1	–	–	–	0	285 ± 30
	2	–	–	–	30000	108 ± 16*
	3	+	50 U/ml, 3 days	4×10^7	0	204 ± 37*
	4	+	50 U/ml, 3 days	4×10^7	30000	9 ± 3**
	5	–	3000 U/ml, 3 days[d]	4×10^7	30000	96 ± 15*
2	6	–	–	–	0	297 ± 30
	7	–	–	–	30000	180 ± 19*
	8	+	50 U/ml, 5 days	1×10^7	30000	0 ± 0**

* $P < 0.05$ compared with the untreated group
** $P < 0.01$ compared with the untreated group
[a] Tumor-draining lymph node cells (1×10^6 cells/ml) were cultured either with or without anti-CD3 mAb (1 μg/ml) and LPS blasts (3×10^6 cells/ml), and followed by a subsequent culture with IL-2 for 3 or 5 days
[b] The injection of IL-2 was started on the same day as when the cultured cells were transferred. IL-2 was i.p. injected twice daily for 4 days
[c] On day 0, B6 mice were i.v. inoculated with 1×10^5 B16 melanoma and, on day 3, the effector cells were i.v. transferred. On day 21 after tumor inoculation, the mice were sacrificed and the numbers of pulmonary metastatic colonies were counted. Each group consisted of seven mice
[d] Normal B6 spleen cells cultured with 3000 U/ml IL-2 for 3 days were used as lymphokine-activated killer cells

Table 2 The antitumor effect of the B16-draining lymph node (*LN*) cells expanded by the culture with anti-CD3 mAb and LPS blasts/IL-2 was specific for the tumor of donor mice

Effector cells		IL-2[c] injected (U)	Mean number of[d] pulmonary metastases ± SEM
The draining LN cells[a] were derived from	Cultured with anti-CD3 mAb[b] and LPS blasts/IL-2		
(–)	(–)	0	299 ± 40
(–)	(–)	30000	182 ± 55
B16-bearing	(+)	30000	0.6 ± 0.5*
MCA-bearing	(+)	30000	154 ± 36**
Untreated	(+)	30000	130 ± 66***

* $P < 0.01$ compared with the untreated group
** $P < 0.05$ compared with the untreated group
[a] The tumor-draining lymph node cells were prepared from B6 mice bearing B16 or MCA for 15 days
[b] The tumor-draining lymph node cells (1×10^6 cells/ml) were cultured with both anti-CD3 mAb (1 μg/ml) and LPS blasts (3×10^6 cells/ml) for 2 days, and with 50 U/ml IL-2 for 5 days
[c] The injection of IL-2 was started on the same day as when the cultured cells were transferred. IL-2 was i.p. injected twice daily for 4 days
[d] On day 0, B6 mice were i.v. inoculated with 1×10^5 B16 melanoma and, on day 3, 1×10^7 effector cells were transferred i.v. On day 28 after tumor inoculation, the mice were sacrificed and the numbers of pulmonary metastatic colonies were counted. Each group consisted of seven mice

and LPS blasts/IL-2 (50 U/ml) for 5 days. The result was that the adoptive transfer of the cultured cells and subsequent i.p. injections of IL-2 were able to eradicate established B16 metastases almost completely (group 8).

The antitumor effect of tumor-draining lymph node cells expanded in the culture with anti-CD3 mAb and LPS blasts/IL-2 were tumor-specific

We determined whether or not the antitumor effect of the cultured T cells is specific for the tumors borne by the donor mice of the tumor-draining lymph node cells. We examined the antitumor activity of the cultured draining lymph node cells from either B16-bearing or MCA-bearing mice against pulmonary metastatic B16 melanoma. Table 2 shows that anti-B16-specific activity could be elicited when the cultured cells derived from the B16-draining lymph node cells were transferred. On the other hand, no anti-B16-specific activity was induced when the cultured MCA-draining lymph node cells were transferred. In addition, Fig. 5 shows that the transfer of 2×10^7 cultured B16-draining lymph node cells, which had been cultured with anti-CD3 mAb and LPS blasts/IL-2, rescued all the mice with the established (on day 3) B16 metastases. The transfer of the cultured MCA-draining lymph node cells did not lead to any antitumor activity against the established B16 metastases. Moreover, we observed that the adoptive transfer of the cultured draining lymph node cells from MCA-bearing mice could not prolong the survival of mice i.v. injected with B16 3 days before (data not shown).

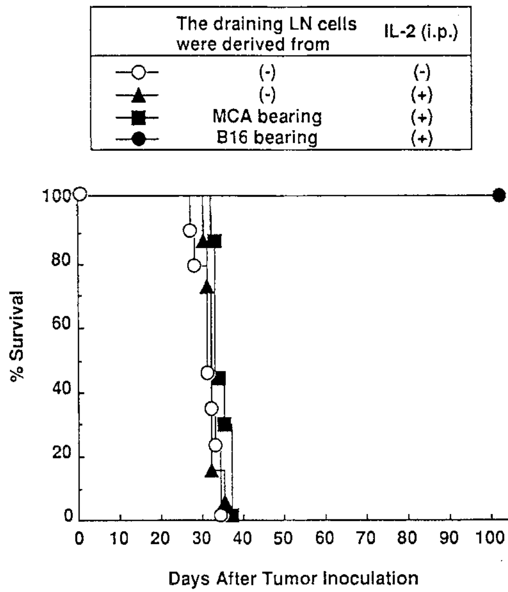


Fig. 5 The survival assay of the mice that were i.v. injected with melanoma and subsequently treated by adoptive immunotherapy. Pulmonary metastases were established by an i.v. injection of 1×10^5 B16 melanoma cells. On day 3, 2×10^7 cultured cells were transferred i.v. The i.p. injections of 30000 U IL-2 were started on day 3 and performed twice daily for the following 4 days. Eight to ten mice were used in each group. The same results were obtained as in the two other experiments. *LN* lymph node

These findings indicate that the antitumor effect of the draining lymph node cells cultured with anti-CD3 mAb and LPS blasts/IL-2 was tumor-specific.

Tumor-specific protective immunity was induced in the mice treated by adoptive immunotherapy

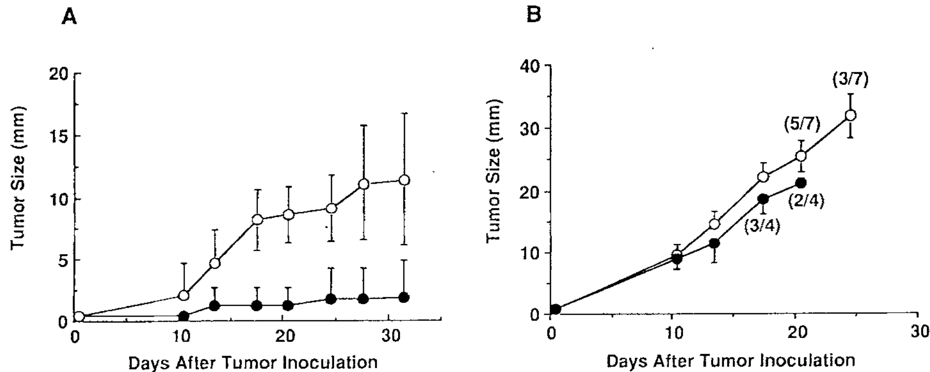
We next determined whether tumor-specific protective immunity was induced in the mice that rejected B16 by the transfer of the cultured B16-draining lymph node cells. Figure 6 shows that the growth of B16 at the rechallenge was significantly suppressed in the B16-rejecting mice in comparison with the naive mice. In addition, there was no difference in the growth of EL-4 between the B16-rejecting mice and the naive mice. We also observed that anti-B16-specific CTL could be induced in the spleen cells from the B16-rejecting mice (data not shown).

Discussion

In this study, we showed that tumor-draining lymph node cells are efficiently activated by both anti-CD3 mAb and LPS blasts, and subsequently expanded by a low dose of IL-2 (200- to 500-fold). We also demonstrated that anti-tumor-specific activity was induced by the adoptive transfer of the tumor-draining lymph node cells expanded by this culture system against established metastatic melanoma. In addition, this treatment cured all B16-bearing mice and these mice also showed tumor-specific protective immunity against B16 at the rechallenge and the induction of B16-specific CTL. We suppose that this culture system may thus be efficient for generating a sufficient number of effector T cells for adoptive immunotherapy against different types of cancer.

The most significant barrier in adoptive immunotherapy against cancers is the difficulty in preparing a sufficient number of antitumor effector cells during a limited period. The availability of recombinant IL-2 enables us to expand T cells or natural killer cells in a short time, and has given rise to the so-called lymphokine-activated killer (LAK) cells [11]. However, in contrast to the in vitro cytotoxic activity, the in vivo efficacy of LAK cells against tumors has been reported to be limited [21]. Therefore, several researchers proposed a culture system in which the pre-sensitized T cells were restimulated in vitro by the autologous tumor cells, to obtain antitumor-specific T cells [25]. This situation led Yoshizawa et al. to report a useful immunotherapy model using tumor-draining lymph node

Fig. 6A, B The tumor-specific protective immunity of the long-term surviving mice mediated by adoptive immunotherapy. **A** On day 57 after the initial i.v. injection of B16 melanoma cells, 2×10^5 B16 cells were s.c. inoculated into either the long-term-surviving B6 mice (● $n = 4$) or the naive B6 mice (○ $n = 7$). **B** On the same day, 2×10^5 EL4 cells were s.c. inoculated into the long-term-surviving B6 mice (● $n = 4$) or the naive B6 mice (○ $n = 7$). The numbers in parentheses indicate the ratio of live mice/total mice. The results are expressed as the mean diameters of the tumors. The error bars represent the SD. The same results were obtained in two similar experiments



cells that were activated with anti-CD3 mAb and followed by a culture with a low dose of IL-2 [30, 31]. The effector cells cultured by their culture system were able to show antitumor activity against established metastases of fibrosarcoma. This culture system is unique in that antitumor-specific effector T cells are induced without any autologous tumor cells. However, the degree of cell expansion is not very large (approximately severalfold), therefore we also tried to develop this culture system further to obtain a large number of effector T cells.

Recently, it was revealed that T cells need several kinds of costimulatory signals for their optimal activation [23, 24]. In addition, it is known that activated B cells express Fc receptor [15] and several kinds of costimulatory molecules, such as B-70, B7-2, intracellular adhesion molecule-1 or heat-stable antigen, on their surface [2, 5, 9, 16]. It therefore occurred to us that we would utilize the costimulatory molecules on LPS blasts for the in vitro culture system in combination with anti-CD3 mAb. In fact, Liu et al. reported that the joint presence of anti-CD3 mAb and activated B cells allows optimal signals for T cells to be delivered [16, 17]. In this study, we demonstrated that the additional presence of LPS blasts stimulated the efficient expansion of naive and tumor-draining lymph node cells in cooperation with the stimulation of anti-CD3 mAb. For clinical application, the number of effector cells is critical for the success of adoptive immunotherapy. We thus suppose that this culture system, utilizing "the two-signal theory", could also provide an effective immunotherapeutic strategy against cancers.

It is still unclear why the antitumor effector T cells effectively expanded in our culture system. One plausible mechanism may be that a coculture with anti-CD3 mAb and LPS blasts results in the binding of the Fc portion of anti-CD3 mAb to Fc receptor on the LPS blasts. As a consequence, such a complex can activate the antitumor CTL or their presursors, which were presensitized in vivo in the tumor-draining lymph nodes. Considering the fact that costimulatory signals alone can enhance the maturation of allo-reactive CTL [1], we suppose that the T cells presensitized in vivo proliferated and matured in response to the optimal stimulation with both anti-CD3 mAb and LPS blasts.

In our culture system, $CD8^+$ T cells expanded preferentially and, as a result, the following possibilities can be considered. The first is that the tumor-bearing states were accompanied by a dysfunction of the $CD4^+$ T cells. In fact, it has been reported that $CD4^+$ T cells in the tumor-bearing mice are suppressed by transforming growth factor β [27, 28]. Therefore, it is likely that the $CD4^+$ T cells had already been suppressed in vivo, so that the in vitro culture resulted in a preferential expansion of $CD8^+$ T cells. An alternative possibility is that the different level of IL-2 receptor expression resulted in a preferential expansion of $CD8^+$ T cells. We observed that the expression level of IL-2 receptor α chain of the lymph node cells was higher on $CD8^+$ T cells than on $CD4^+$ T cells after culture with both anti-CD3 mAb and LPS blasts for 1 day (unpublished data). The latter possibility is now under investigation.

Recent reports revealed that antitumor activity could be elicited by the cooperation of $CD4^+$ and $CD8^+$ T cells and the helper function of $CD4^+$ T cells could be substituted by the administration of IL-2 [10]. The adoptive transfer of the effector cells prepared by our culture system without i.p. injections of IL-2 showed only a low level of antitumor activity against metastatic melanoma (Table 1). We thus suppose that the role of the injected IL-2 is to maintain the in vivo survival of the transferred T cells in the tumor-bearing hosts.

Recent findings suggest that costimulatory molecules are essential for the induction of effector T cells effective for tumor eradication in vivo [4]. Several researchers have reported that melanoma cells transfected with B7 molecules, which are representative of costimulatory molecules, could be rejected in syngeneic mice in a $CD4^+$-T-cell-independent manner [3, 29]. These lines of evidence suggest the value of making use of the costimulatory molecules to enhance the antitumor immunity in vivo. In this paper, we have for the first time demonstrated that activated B cells were useful in the in vitro culture system for the antitumor T cells. We would like to emphasize that the culture system described is clinically simple since we can easily prepare activated B cells from patients and obtain the tumor-draining lymph node cells perioperatively. We are now developing a plan to apply this culture system in clinical trials.

Acknowledgements We specially thank Dr. J. A. Bluestone for providing the hybridoma 145-2C11. We would also like to thank Dr. B. T. Quinn for his comments on this manuscript. This work was supported in part by a grant from the Ministry of Education, Science and Culture. This work also received financial support from the Comprehensive 10-year Strategy for Cancer Control.

References

1. Azuma M, Cayabyab M, Muck D, Phillips J, Lanier LL (1992) CD28 interaction with B7 costimulates primary allogeneic proliferative responses and cytotoxicity mediated by small, resting T lymphocytes. J Exp Med 175: 353
2. Azuma M, Ito D, Yagita H, Okumura K, Phillips JH, Lanier LL, Somoda C (1993) B70 antigen is a second ligand for CTLA-4 and CD28. Nature 366: 76
3. Chen L, Ashe S, Brady WA, Hellström I, Hellström KE, Ledbetter JA, McGowan P, Linsley PS (1992) Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocytes molecules CD28 and CTLA-4. Cell 71: 1093
4. Chen L, Linsley PS, Hellström KE (1993) Costimulation of T cells for tumor immunity. Immunol Today 14: 483
5. Damle NK, Klussman K, Linsley PS, Aruffo A, Ledbetter JA (1992) Differential regulatory effects of intercellular adhesion molecule-1 on costimulation by the CD28 counter-receptor B7. J Immunol 149: 2541
6. Fearon ER, Pardoll DM, Itaya T, Golumbek P, Levitsky HI, Simons JW, Karasuyama H, Vogelstein B, Frost P (1990) Interleukin-2 production by tumor cells bypasses T helper function in the generation of an antitumor response. Cell 60: 397
7. Fraser JD, Irving BA, Crabtree GR, Weiss A (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. Science 251: 313

8. Freeman GJ, Freeman AS, Segil JM, Lee G, Whiteman JF, Nadler LM (1989) B7, a new member of the Ig superfamily with unique expression on activated and neoplastic B cells. J Immunol 143: 2714
9. Freeman GJ, Gribben JG, Boussiotis VA, Ng JW, Restivo VA Jr, Lombard LA, Gray GS, Nadler LM (1993) Cloning of B7-2: a CTLA-4 counter-receptor that costimulates human T cell proliferation. Science 262: 909
10. Greenberg PD (1991) Adoptive T cell therapy of tumors: mechanisms operative in the recognition and elimination of tumor cells. Adv Immunol 49: 281
11. Grimm EA, Mazumder A, Zhang HZ, Rosenberg SA (1982) Lymphokine-activated killer cell phenomenon. Lysis of natural killer-resistant fresh solid tumor cells by interleukin-2 activated autologous human peripheral blood lymphocytes. J Exp Med 155: 1823
12. Jenkins MK, Taylor PS, Norton SD, Urdhl KB (1991) CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. J Immunol 147: 2461
13. Kurosawa S, Matsuzaki G, Harada M, Ando T, Nomoto K (1993) Early appearance and activation of natural killer cells in tumor-infiltrating lymphoid cells during tumor development. Eur J Immunol 23: 1029
14. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter JA (1991) CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med 174: 561
15. Liu Y, Janeway CA Jr (1991) Microbial induction of co-stimulatory activity for CD4 T-cell growth. Int Immunol 3: 323
16. Liu Y, Jones B, Aruffo A, Sullivan KM, Linsley PS, Janeway CA Jr (1992) Heat-stable antigen is a costimulatory molecule for CD4 T cell growth. J Exp Med 175: 437
17. Liu Y, Jones B, Brady W, Janeway CA Jr, Linsley PS (1992) Co-stimulation of murine CD4 T cell growth: cooperation between B7 and heat-stable antigen. Eur J Immunol 22: 2855
18. Nores GA, Dohi T, Taniguchi M, Hakomori S (1987) Density-dependent recognition of cell surface GM3 lactone as a possible immunogen; requirements for tumor associated antigen and immunogen. J Immunol 139: 3171
19. North RJ (1982) Cyclophosphamide-facilitated adoptive immunotherapy of an established tumor depends on elimination of tumor-induced suppressor T cells. J Exp Med 5: 1063
20. Rosenberg SA, Spiess PJ, Lanreniere R (1986) A new approach to the adoptive immunotherapy of cancer with tumor infiltrating lymphocytes. Science 233: 1318
21. Rosenberg SA, Lotze MT, Muul LM, Chang AE, Avis FP, Leitman S, Linehan M, Robertson C, Lee RE, Rubin JT, Seipp CA, Simpson CG, White DE (1987) A progress report on the treatment of 157 patients with advanced cancer using lymphokine-activated killer cells and interleukin-2 or high-dose interleukin-2 alone. N Engl J Med 316: 889
22. Rosenberg SA, Packard BS, Aebersold PM, Solomon D, Topalian SL, Toy ST, Simon P, Schwartzentruber D, Wei JP, White DE (1988) Immunotherapy of patients with metastatic melanoma using tumor-infiltrating lymphocytes and interleukin-2: preliminary report. N Engl J Med 319: 1676
23. Schwartz RH (1989) Acquisition of immunological self-tolerance. Cell 57: 1073
24. Schwartz RH (1990) A cell culture model for T lymphocyte clonal anergy. Science 248: 1349
25. Shu S, Chou T, Rosenberg SA (1986) In vitro sensitization and expansion with viable tumor cells and interleukin 2 in the generation of specific therapeutic effector cells. J Immunol 136: 3891
26. Shu S, Chou T, Rosenberg SA (1987) In vitro differentiation of T cells capable of mediating the regression of established syngeneic tumors in mice. Cancer Res 47: 1354
27. Tada T, Sano H, Sato S, Shima J, Fujiwara H, Hamaoka T (1990) Immune dysfunction expressed selectively on L3T4+ T cells in the tumor-bearing state. J Leukoc Biol 47: 149
28. Tada T, Ohzeki S, Utsumi K, Takiuchi H, Muramatsu M, Li XL, Shimizu J, Fujiwara H, Hamaoka T (1991) Transforming growth factor-β-induced inhibition of various phenotypes and functions and its relevance to immunosuppression in the tumor-bearing state. J Immunol 146: 1077
29. Townsend SE, Allison JP (1993) Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. Science 259: 368
30. Yoshizawa H, Chang AE, Shu S (1991) Specific adoptive immunotherapy mediated by tumor-draining lymph node cells sequentially activated with anti-CD3 and IL-2. J Immunol 141: 729
31. Yoshizawa H, Chang AE, Shu S (1992) Cellular interactions in effector cell generation and tumor regression mediated by anti-CD3/interleukin 2-activated tumor-draining lymph node cells. Cancer Res 52: 1129