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Activation by Anti-CD3 of Tumor-Draining Lymph Node Cells for Specific Adoptive Immunotherapy[1]

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Lymph nodes draining progressive tumors contain tumor-sensitized but not functional preeffector T lymphocytes. These cells can acquire antitumor reactivity after stimulation with tumor cells and interleukin-2 (IL-2). We demonstrated here that, in the absence of tumor cells, preeffector cells could be stimulated and expanded by sequential culture with anti-CD3 monoclonal antibody and IL-2. The adoptive transfer of such activated cells mediated immunologically specific reductions of established pulmonary metastases. The therapeutic effects could be enhanced by the administration of IL-2. This activation represents a secondary immune response because effector cells could be generated only from tumor-draining but not from normal or adjuvant-stimulated lymph nodes. Furthermore, treatment of advanced metastases with these cells resulted in prolongation of survival and cure of the disease. Thus, anti-CD3 may serve as a universal reagent for activating tumor-sensitized T lymphocytes for cancer therapy.

INTRODUCTION

The adoptive transfer of immune T lymphocytes into tumor-bearing animals can mediate the regression of established primary and metastatic tumors (l-3). The most critical aspect for the successful clinical application of adoptive immunotherapy is the identification of functional immune cells from cancer patients. Because the putative immune response during tumor progression is weak, isolation of a sufficient number of immune effector cells must depend on the ability to boost this reactivity by in *vitro* manipulations. Using syngeneic animal tumors, we have recently described a method whereby lymph node (LN)[3] cells draining progressive tumor could be stimulated and expanded to acquire therapeutic efficacy (3-5). To accomplish this, LN cells had to be antigenically stimulated by the tumor of origin in the presence of interleukin-2 (IL-2). These observations have prompted us to evaluate similarly generated tumor-sensitized T lymphocytes for the treatment of humans with advanced cancer. Although this method is theoretically and technically feasible, the requirement of large numbers of autologous tumor cells has restricted its application in many cases. Antigenic stim-

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[3] Abbreviations used: LN, lymph node(s); IL-2, interleukin-2; IL-2R, IL-2 receptor; TCR, T cell antigen receptor; mAb, monoclonal antibody(ies); HBSS, Hanks' balanced salt solution.

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ulation of T lymphocytes involves the T cell receptor (TCR) which is noncovalently associated with a cluster of low MW proteins commonly referred to as CD3 (6). Considerable evidence indicates that the CD3 structure serves to transduce the activation signals consequential to the binding of antigen to TCR (7). Indeed, in the absence of the antigen, mAb reactive to CD3 molecules have been shown to induce T cell proliferation and lymphokine secretion (8, 9). In the present study, we show that tumor-draining LN cells proliferated when stimulated with an anti-CD3 mAb and subsequently cultured in IL-2. More significantly, such activated cells were highly effective in mediating the immunologically specific regression of established tumor metastases.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and were used at the age of 10 weeks or older.

Tumors. The MCA 106 tumor is a 3-methylcholanthrene-induced fibrosarcoma syngeneic to B6 mice (10). It was maintained *in vivo* by serial SC transplantation of cryopreserved tumor samples and used for the current study in the fourth to seventh transplantation generation. Other similarly induced MCA 102 and MCA 105 sarcomas of B6 origin served as specificity controls. Single cell suspensions were prepared from solid tumors by digestion in 40 ml of HBSS (GIBCO, Grand Island, NY) containing 4 mg of DNase, 40 mg of collagenase, and 100 U of hyaluronidase (Sigma Chemical Co., St. Louis, MO) for 3 hr at room temperature.

For initiating solid tumor growth, B6 mice were inoculated SC in the footpad with 8-10 X 10^5 viable tumor cells in 0.05 ml of HBSS. Twelve to 14 days later, when footpad thickness increased to approximately 8 mm, tumor-draining popliteal LN were harvested and single cells were prepared mechanically.

Anti-CD3 mAb. A hamster mAb, 145-2C11, directed against the CD3-ϵ chain of the murine TCR/CD3 complex was produced and kindly provided by Dr. Jeffrey A. Bluestone, University of Chicago (9). The mAb was harvested as ascites fluid following inoculation of sublethally irradiated (500 R) DBA/2 mice with the hybridoma cells. Ab was then partially purified by 50% ammonium sulfate precipitation. The hamster IgG content of the preparation was determined by ELISA.

IL-2. Human recombinant IL-2 was kindly supplied by the Cetus Corp. (Emeryville, CA). The biological and biochemical activities of this material have been described (11). Purified material had a specific activity of 3.0 X 10^6 U/mg. In our assay 1 U of Cetus IL-2 is equivalent to 2-3 U of the standard of the Biological Response Modifiers Program of the National Cancer Institute.

Activation of LN cells with anti-CD3/IL-2. Tumor-draining LN cells were activated by incubating 6-10 X 10^7 cells in a 75-cm^2 tissue culture flask containing 30 ml of complete medium (12) with 1 μg/ml of anti-CD3 at 37°C in a 5% CO_2 atmosphere for 2 days. The cells were harvested, washed, and further cultured at 4-6 x 10^5 cells/well in 2 ml of complete medium containing 10 U/ml of human recombinant IL-2 in 24-well plates for 3 days. The resulting cells were resuspended in HBSS for adoptive immunotherapy.

Adoptive immunotherapy. B6 mice were injected iv with 8-10 x 10^5 MCA 106 tumor cells suspended in 1.0 ml of HBSS to initiate pulmonary metastases. On Day 3, anti-CD3/IL-2-activated LN cells were given iv. In most experiments, mice receiving cells were also given ip injections of IL-2 to enhance the antitumor efficacy of the

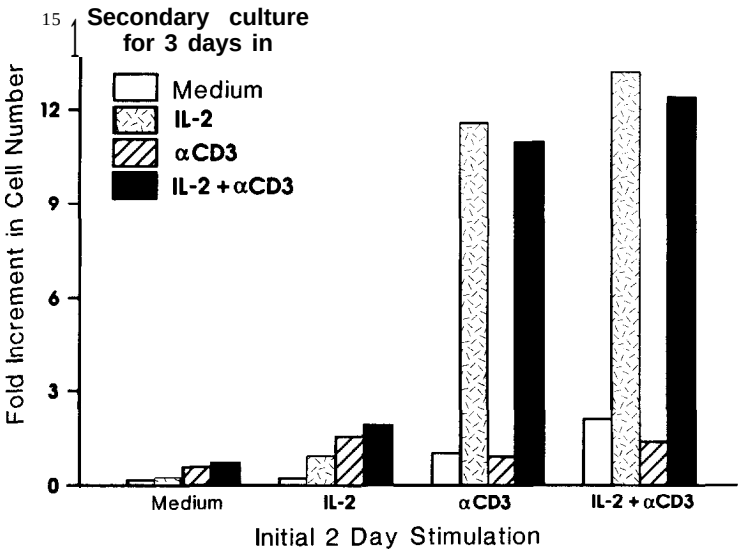

FIG. 1. Proliferative response of tumor-draining LN cells from mice bearing intrafootpad MCA 106 tumors for 14 days. Single cell suspensions prepared from popliteal LN were cultured at 6 X 10^7 cells in 30 ml of complete medium containing I μg/ml of anti-CD3, 10 U/ml of IL-2. or both for 2 days. Harvested cells were washed and replated in 24-well culture plates at 6 X 10^5 cells/well in the presence of anti-CD3, IL-2, or both for an additional 3 days. It appeared that stimulation with anti-CD3 followed by expansion in IL-2 was necessary for maximal proliferation.

transferred cells. Between Days 21 and 27 after tumor induction, the mice were randomized and sacrificed for enumeration of pulmonary tumor nodules as described (13). Metastatic foci too numerous to count were assigned an arbitrary value of 250 since this was the greatest number of tumor nodules that could be reliably enumerated. Each treatment group consisted of at least five animals. Statistical analyses were performed by the Wilcoxon rank sum test. A two-sided P value of ≤ 0.05 was considered a significant difference between two groups.

In some experiments, mice with advanced pulmonary metastases (10 days after tumor initiation) were treated with anti-CD3/IL-2-activated cells in conjunction with the administration of exogenous IL-2. Therapeutic efficacy was evaluated by the survival of treated animals.

RESULTS AND DISCUSSION

The therapeutic efficacy of adoptive immunotherapy is proportional to the numbers of immune effector cells transferred. Therefore, we initially examined the conditions under which maximal cellular proliferation with concurrent activation of effector cells could be induced by anti-CD3. Tumor-draining LN cells stimulated with anti-CD3 mAb at a concentration of 1 μg/ml for 2 days did not result in significant proliferation. However, these anti-CD3-stimulated cells responded to a low concentration (10 U/ml) of IL-2. After 3 days in the presence of IL-2, more than a IO-fold increase in cell numbers was observed (Fig. 1). This cellular proliferation was not induced when LN cells were cultured in either anti-CD3 or IL-2 alone for a total of 5 days. In addition,

TABLE 1

Conditions and Requirements for Generation of Antitumor Effector Cells by the Method of Anti-CD3/IL-2 Activation

Source of LN cells[a]	*In vitro* activation	*In vivo* IL-2	Adoptive immunotherapy: Mean No. of pulmonary MCA 106 metastases (SEM) Expt 1	Expt 2
		-	*250*	*250*
		+	*250*	*240 (9)*
Tumor-draining	Anti-CD3/IL-2	+	6 (1)[b]	2 (1)[b]
Tumor-draining	Con A/IL-2	+	132 (17)	191 (33)
Tumor-draining	IL-2/IL-2	+		190 (30)
Corynebacterium parvum stimulated	Anti-CD3/IL-2	+	216 (22)	
Normal mesenteric	Anti-CD3/IL-2	+	*205 (29)*	*237* (11)

[a] Popliteal LN cells draining the progressively growing MCA 106 tumors for 12 days were cultured in 1 μg/ml of anti-CD3, 2 μg/ml of Con A, or 10 U/ml of IL-2 for 2 days followed by expansion in 10 U/ml of IL-2 for 3 days. Normal mesenteric LN cells and LN cells draining injections of 100 μg of *C. parvum* for 12 days were activated by anti-CD3/IL-2. All cells were tested for *in vivo* antitumor effects in adoptive immunotherapy of 3-day established pulmonary MCA 106 metastases. Each mouse received iv 6 x 10^6 activated cells. IL-2 (15,000 U, twice daily) was given ip from Day 3 to Day 6.

[b] Significantly different when compared with groups receiving no treatment or IL-2 only.

the proliferation response was not enhanced even if both anti-CD3 and IL-2 were present during the two stages of culture.

By phenotype analysis, freshly harvested tumor-draining LN contained 37 ± 9% Thy-l+ lymphocytes with 18 ± 2% of $CD4^+$ ($L3T4^+$) and 14 ± 1% of $CD8^+$ ($Lyt\text{-}2^+$) cells. There was little or no detectable IL-2 receptor (IL-2R) present on these cells. However, after 2 days of activation with anti-CD3, recovered cells were virtually all Thy-l+ (296%) and consisted of 32 ± 6% $CD4^+$ and 59 ± 8% $CD8^+$ cells. At this stage, IL-2R expression became evident as revealed by flow cytometry. The greatest cellular proliferation occurred when anti-CD3-stimulated LN cells were cultured in IL-2 for an additional 3 days. During this time, cell numbers doubled every 24 hr. $CD8^+$ cells appeared to respond more vigorously than $CD4^+$ cells because the IL-2-expanded cell population contained 75 ± 9% $CD8^+$ and 24 ± 7% $CD4^+$ cells.

The antitumor efficacy of such activated cells was first examined in the adoptive immunotherapy of 3-day established pulmonary MCA 106 metastases. In several preliminary experiments, the transfer of 2 X 10^7 activated cells alone was capable of reducing the numbers of pulmonary metastases from ⩾250 in the nontreated groups to an average of 4 ± 2. The antitumor reactivity of these cells could be further enhanced by the concomitant administration of exogenous IL-2 (15,000 U, twice daily) for 4 days. In the same experiments, the transfer of as few as 6 X 10^6 activated cells in conjunction with IL-2 injections reduced pulmonary metastases to 2 ± 1. Since treatment with IL-2 alone did not have any therapeutic effects, these results suggest that the administered IL-2 acted on the transferred cells probably by promoting their survival and function *in vivo.*

TABLE 2

Specificity of Adoptive Immunotherapy Mediated by Anti-CD3/IL-2-Activated Tumor-Draining LN Cells

Treatment		Mean No. pulmonary metastases (SEM)[b]		
Source of draining LN[a]	IL-2	MCA 106	MCA 105	MCA 102
—	—	240 (10)	250	250
	+	216 (19)	250	250
MCA 106	+	2 (1)[c]	250	217 (28)
MCA 105	+	246 (4)		
MCA 102	+	250		

[a] Popliteal LN cells draining the progressively growing MCA tumors in the footpad for 14 days were activated by anti-CD3/IL-2 as described in Table 1.

[b] Pulmonary metastases were induced by iv injections of 3 x 10^5 (MCA 102), 3 x 10^5 (MCA 105), or 10^6 (MCA 106) tumor cells. The protocol for adoptive immunotherapy was as described in Table 1. except that 2 x 10^7 activated cells were given to each mouse.

[c] Significantly different when compared with groups receiving no treatment or IL-2 only.

The anti-CD3 mAb has reactivity toward all T lymphocytes and we found that the activation of normal LN cells induced an equivalent cell proliferation. Therefore, we examined the *in vivo* and *in vitro* conditions and requirements that were necessary for the generation of therapeutically effective cells. Normal mesenteric LN cells and cells prepared from popliteal LN draining intrafootpad injections of 100 μg of *Corynebacterium parvum* (Burroughs Wellcome Co., Research Triangle Park, NC) were activated by anti-CD3 and IL-2 for 5 days. These cells as well as anti-CD3/IL-2-activated MCA 106 tumor-draining LN cells were tested for *in vivo* antitumor effects (Table 1). Neither activated normal LN cells nor adjuvant-stimulated LN cells demonstrated significant antitumor effects. These results clearly indicate the requirement of a critical sensitization during progressive tumor growth. On the other hand, the transfer of as many as 5 X 10' freshly harvested tumor-draining LN cells with or without concomitant IL-2 treatment did not demonstrate antitumor reactivity (data not shown). Thus, the *in vitro* activation with anti-CD3/IL-2 represents a secondary immune response which activates previously sensitized preeffector cells. This is in contrast to the mitogenic effects of anti-CD3 which do not exhibit antigenic specificity. Also examined were conditions under which preeffector cells could develop into therapeutic effector cells *in vitro.* In this part of the experiment, MCA 106 tumor-draining LN cells were cultured in anti-CD3 (1 μg/ml), Con A (2 μg/ml), or IL-2 (10 U/ml) for 2 days and harvested cells were expanded in IL-2 (10 U/ml) for 3 days. As also presented in Table 1, cells activated by Con A or cultured in IL-2 only failed to develop into antitumor effector cells. It thus appears that the interaction of anti-CD3 with preeffector cells, although not antigenically specific, provides essential signals to trigger their maturation into functional therapeutic cells. In previous studies (4, 5), we found that tumor-draining LN cells could be stimulated *in vitro* by specific tumor cells to differentiate into immune effector cells. It is therefore likely that anti-CD3 mimics the antigenic stimulation in the secondary activation.

Numerous reports have demonstrated that the interaction of anti-CD3 with T lymphocytes induced proliferation, secretion of various lymphokines/cytokines, up-regulation of the expression of IL-2R, and/or the generation of cytotoxic effector cells (8,

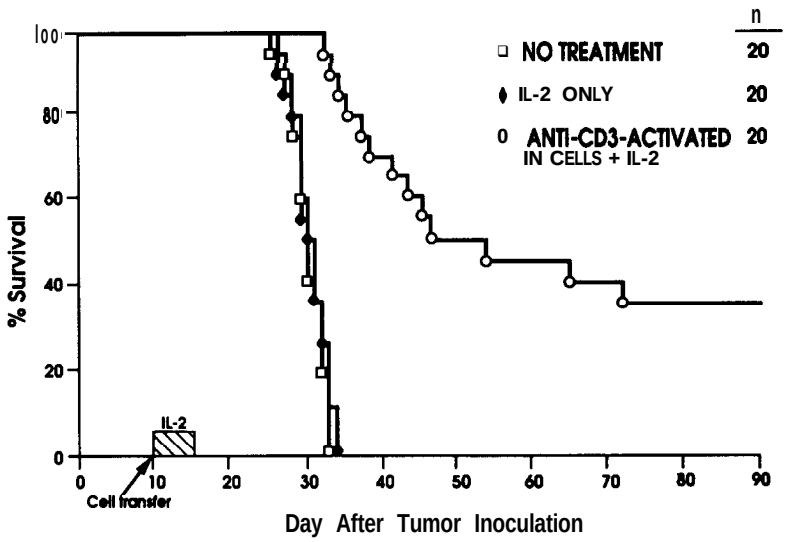

FIG. 2. Adoptive immunotherapy of mice with advanced pulmonary MCA 106 metastases. This figure summarizes combined results of two independent but identically designed experiments. Pulmonary metastases were induced by the iv injection of 10^6 MCA 106 tumor cells. Ten days later, when metastatic nodules were clearly visible, therapy was instituted by iv infusion of 8 X 10' anti-CD3/IL-2-activated tumor-draining LN cells (prepared as described in Table 1). The dose of IL-2 was 15,000 U given ip twice daily, for 5 days. Treatment with activated LN cells and IL-2 resulted in a significant prolongation of survival ($P < 0.05$, Wilcoxon rank sum test).

9, 14). None of these reactivities represent antigen-specific events, probably due to the ubiquitous presence of CD3 molecules on all T lymphocytes. However, it is also known that anti-CD3 is capable of activating specific cytotoxic T cells in the absence of nominal antigens (15). The results of the preceding experiments thus suggest that an element of anti-CD3-T cell interactions might reflect stimulation of tumor antigen-sensitized preeffector cells. We therefore examined the specificity of adoptive immunotherapy mediated by anti-CD3/IL-2-activated cells. In the experiment summarized in Table 2, it is clear that effector cells generated from MCA 106 tumor-draining LN cells mediated the regression of the MCA 106 tumor but not that of the antigenically distinct MCA 102 or MCA 105 tumor. At the effector cell level, the regression of the MCA 106 tumor could be mediated only by cells generated from MCA 106 but not MCA 102 or MCA 105 tumor-draining LN. Moreover, anti-CD3/IL-2-activated MCA 102 and MCA 105 tumor-draining LN cells mediated the regression of their respective tumors (data not shown). Therefore, the specificity of antitumor reactivity was apparently determined at the preeffector cell level, i.e., during progressive tumor growth. These results are consistent with the notion that the anti-CD3/IL-2 activation is a secondary immune reaction. In this situation, the anti-CD3 mAb serves a function similar to that of the specific antigen for activation of immunologically committed preeffector T cells. Despite their ability to mediate tumor regression *in vivo, we* have consistently found that the effector cells did not exhibit cytotoxic effects *in vitro* in the standard 4-hr chromium release assay (data not shown). This finding is intriguing and has prompted us to investigate the mechanisms of tumor eradication mediated by these noncytolytic effector cells.

The adoptive immunotherapy experiments described thus far involved the treatment of 3-day established pulmonary metastases. At this stage of disease, metastases were only microscopically evident, To further evaluate the therapeutic efficacy of anti-CD3/IL-2-activated cells, mice with advanced metastases were treated with these cells. Pulmonary metastases were initiated by iv injection of mice with MCA 106 tumor cells. Adoptive immunotherapy was instituted 10 days later when tumor nodules on the surface of the lung became clearly visible. The pooled results of two independent experiments are summarized in Fig. 2. Mice that were not treated or that were treated for 5 days with IL-2 (15,000 U, twice daily) succumbed to the growing tumor with median survival times of 30 and 3 1 days, respectively. Mice treated with 8 X 10^7 anti-CD3/IL-2-activated tumor-draining LN cells plus exogenous IL-2 showed a prolonged median survival time of 50 days, and 7 of 20 treated animals were tumor-free for >90 days. The high therapeutic efficacy of these activated cells provides a rationale for the use of similarly activated cells from cancer patients for adoptive immunotherapy.

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