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    A limitation of adoptive immunotherapy is the poor survival and tumor localization of activated T cells after infusion. This problem arises in part from the polarized, adhesive form of activated T cells, which makes them prone to embolize in microvasculature. We hypothesized that transient inhibition of T cell polarization, induced just before infusion, will improve the survival and circulation of activated T cells. We found previously that T cells can be depolarized by the myosin light-chain kinase inhibitor ML-7. During the first project year we developed an ML-7 pretreatment protocol which depolarized activated T cells for 1 - 2 hr, then allowed them to recover normal cytotoxicity and proliferation within 24 hr. Initial experiments indicated that this protocol increased fourfold the homing of ErbB2-specific T cells to the ErbB2-expressing murine mammary tumor D2F2/E2. We have now confirmed this finding and tested whether the improved localization increased therapeutic effects. ML-7 pretreatment was found neither to enhance nor reduce the tumor-delaying effects of T cell infusion. This work is the first proof that cytoskeletal alteration of T cells can improve their trafficking behavior. Our first test, however, could not confirm that this strategy alone is enough to enhance adoptive immunotherapy.

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INTRODUCTION

Note: This was originally to have been the Final Report of a two-year project. A no-cost extension has been granted to allow completion of some delayed experiments. This report is therefore the second Annual Report.

Relapse after surgery and chemotherapy is a major cause of therapeutic failure in breast cancer patients. Vaccination against tumor antigens is a strategy which holds great promise for preventing recurrence, but it requires an intact immune system [1]. Immunotherapy by adoptive transfer of activated T cells is potentially an excellent way to prevent local and metastatic tumor growth during the period of immunological depletion which follows intensive radiotherapy and chemotherapy [2]. Patient T cells are stimulated in vitro to a peak of population size and anti-tumor activity and infused back into the patient. A serious limitation on adoptive immunotherapy is the poor ability of the infused T cells to circulate properly. A contributing factor to this problem is the morphological and behavioral phenotype of the cells. T cells normally circulate in a state of rest or early activation, in which they are round, smooth, and nonadhesive. Activation in vitro pushes them into a polarized, spiky, adhesive and highly motile form which they never normally achieve until after extravasation. When infused in this polarized state, T cells tend to clump together and to lodge in microvasculature. Many are trapped, embolized, and damaged during their initial passages through pulmonary microvasculature, and are later destroyed in the liver and spleen. Relatively few transferred cells survive to enter tumor masses and secondary lymphoid organs [3]. The polarized, adhesive phenotype of activated T cells is created by the actin-myosin cytoskeleton, which, in activated T cells, is programmed to produces appendages and to hold adhesion receptors in an activated state [4,5]. We have found previously that activated T cells can be rendered temporarily round, smooth, and nonadhesive in vitro by treatment with pharmacological inhibitors of myosin function. We hypothesized that pretreatment of activated T cells with myosin inhibitors prior to infusion will allow the cells to avoid initial trapping in lung microvasculature and circulate widely before regaining the polarized, tissue-invading phenotype. During the first year of this project we completed the first steps of testing this overall hypothesis in a murine model of breast cancer. T cells were fluorescently labeled and tracked in vivo. Initial results described in the first annual report indicated that T cells treated with a myosin inhibitor did indeed show significantly lower initial lung localization and greater longer-term localization in tumors. Furthermore, the proliferative and cytotoxic activity of the cells rapidly recovered from the inhibition of myosin function. During the past year we repeated and confirmed the localization results. We also determined whether the increased lymphocyte localization in tumors was sufficient to improve outcome in a mammary tumor immunotherapy model.

BODY:

Note: The Aims and Tasks referred to below are not those of the original Statement of Work but rather the revised Statement of Work approved January 2002.
Aim 1: To test the hypothesis that temporary depolarization of activated T cells by pharmacological myosin inhibitors will increase the percentage of cells localizing in tumor masses and secondary lymphoid organs after adoptive transfer.

Task 1: "Determine effects of myosin-inhibiting drugs on in vivo T cell localization."

Task 1a and b: . Immunize mice; obtain and specifically expand ErbB2-specific T cell populations.

These tasks were completed during the first year of work and summarized in the first annual report. The results will be summarized in some detail, though, because they are necessary for understanding the past year's work.

Task 1a and b RESULTS:

The procedure for producing murine T cells specific for human ErbB2 specific was developed by a colleague, Wei-Zen Wei, who provided all required cell lines. The tumor used in this study was D2F2/E2, a mammary adenocarcinoma which arose from a spontaneous mammary hyperplasia of a BALB/C mouse and which was stably transfected to express full-length human ErbB2 [6]. Syngeneic BALB/c mice received bilateral s.c. flank injections of 5 X 10(5) irradiated D2F2/E2 cells. This was repeated 4 weeks later, and after an additional 4 weeks draining lymph nodes were excised. ErbB2-specific lymphocytes from these nodes were specifically expanded by culture on irradiated cells from a syngeneic fibroblastoid cell line engineered to stably express human ErbB2 as well as K(d), IA(d)β, and the costimulator B7.1 [7]. This stimulator line will be referred to as "3T3/E2". The T cells were thinned and fed every 2 days with DME containing 10%FCS, 5U IL-2/ml and 10 ng/ml IL-7, and restimulated with irradiated 3T3/E2 every 14-20 days.

After two rounds of stimulation, the lymphocyte population reached a stable phenotype of >98% CD3+, of which typically 55-60% were CD4+, and 45-50% CD8+. After 3 simulations, lymph node cells from 12 mice could be expanded to up to 1.2 X 10(8) cells. They were used in experiments from 7 - 10 days after their last stimulation. At this point, the cells typically exerted approximately 50% specific cytotoxicity against D2F2E2 at an E:T ratio of 50:1, 35% at 25:1, and 10% at ratio of 10:1, as determined by chromium release assay (not shown). Cytotoxicity vs. the unrelated syngeneic mammary tumor 410.4 was <5%.

Task 1c. Determine effects of myosin inhibiting drugs on localization of T cells in mice bearing subcutaneous tumors. Whole-tissue tracking studies, nearly completed during the first year, have now been finished.

Task 1c METHODS, part 1

Depolarization strategy. In the ErbB2-specific T cell cultures mentioned above, greater than 90% of cells displayed polarized elongated form, with complex anterior and posterior appendages. They were highly adhesive and prone to form clumps. Both appendage formation and integrin function are known to be dependent on an intact and
normally functioning cytoskeleton. We found previously that chemical inhibitors of the contractile action of myosin can temporarily abolish appendages and adhesive function in activated lymphocytes and polarized leukemia cells. During the first year of this project we developed the depolarization protocol for the ErbB2-specific T cell cultures. The cells were treated 30 min. with the myosin light-chain kinase inhibitor ML-7 (Calbiochem, 75 uM) followed by three washes. As summarized in the last annual report, this treatment rendered the cells spherical, appendage free, and nonadhesive for a period of 30 - 60 min at 37°C. There was no loss of viability. Recovery of polarity and adhesiveness was nearly complete by 2 - 4 hr after treatment, and cytotoxicity, polarity, and antigen-stimulated proliferation were fully recovered by 24 hr.

Lymphocyte localization experiments. Subcutaneous s.c. D2F2/E2 tumors were allowed to grow to approx. 10 mm diameter (0.4 - 0.6 g), a process which took 3-4 weeks.

The green fluorescent tracking dye CFSE ("Cell Tracker", Molecular Probes) was used to label ErbB2-specific T cells before infusion into these tumor-bearing mice. In pilot studies it was determined that a concentration of 1 uM CFSE conferred sufficient fluorescent brightness to track the cells for at least 48 hr while not reducing the ability of the cells to exert cytotoxicity or to migrate into collagen matrix in vitro.

T cells were treated with ML-7 or DMSO vehicle control and washed as described above, and then infused into D2F2/E2-bearing mice via tail vein, 2 X 10^7 cells/0.5 ml HBSS per mouse. At 30 min, 2 hr, 24 hr, and 48 hr, tissues were harvested, weighed, and dispersed into single cell suspension either by physical disruption (for lymph nodes and spleen) or by a standardized protocol of digestion with Liberase (a commercial collagenase blend), elastase, and DNase. The resulting single-cell suspensions were fixed and measured aliquots were analyzed by flow cytometry for both frequency and absolute numbers of viable CFSE-labeled lymphoid cells.

**Task 1c RESULTS, part 1**

As described in detail in the last annual report, prompt and transient localization of labeled cells is an indicator of trapping in the pulmonary microvasculature. We found that trapping of ErbB2-specific T cells in the lung was reduced eight-fold by pretreatment with ML-7 (p \leq 0.05). Localization of T cells in s.c. tumors was increased by > 4-fold over controls at both 24 and 48 hr. This represented approximately 1% of cells infused. Localization in lymph nodes and spleen showed slight but not significant increase for the ML-7-treated populations. The results are consistent with the concept that transient depolarization of the activated T cells reduced their trapping and damage in the lung and thus increased the number of cells able to circulate and eventually reach tumor sites.

To continue this task, we have now determined whether the ML-7-induced increase in T cell localization in tumors was sufficient to produce a measurable improvement in therapeutic effect. This study was the main accomplishment of the past year of work.

**Task 1c METHODS, part 2.**
**Effector cells.** ErbB2-specific T cells for adoptive immunotherapy were grown as described above.

**Tumors.** D2F2/E2 tumors were initiated s.c. in the flanks of mice, bilaterally, as described above, but were allowed to grow only for 14 days before the start of an experiment, at which point they had become barely palpable. This time point was selected because our hypothesis requires the presence of a mass of tumor cells for the T cells to infiltrate; and because a relatively small tumor burden is more likely to respond to therapy.

In the pilot study, designed to establish therapeutic protocol, mice received i.v. tail vein infusions of 0.5 ml HBSS either alone or containing untreated T cells at doses ranging from 1 to 5 X 10^7 cells per mouse. For experiments determining the effects of ML-7 on therapy, infusions of 3 X 10^7 were given. Mice were divided randomly into three groups: "CONTROL" mice received a tail-vein i.v. infusion of 0.5 ml HBSS. "CELLS + VEH" mice received 0.5 ml HBSS containing T cells which had been treated with vehicle (DMSO) for 30 min, washed 3X, and suspended in HBSS. "CELLS + ML-7" mice received T cells which had been treated 30 min with 75 uM ML-7, washed three times and resuspended in HBSS. Beginning at 7 days post-treatment, tumors were measured with calipers at weekly intervals. Two perpendicular measurements of diameter were made and the average value for each tumor will be referred to as "mean tumor diameter" [6]. Three experiments were performed, with 2 mice in each experimental condition in each experiment. ANOVA (Instat, Graphpad) was used to detect significant treatment-induced differences in mean tumor diameter. When differences were found, ANOVA was followed by paired t test with Bonferroni correction for multiple comparisons.

**Task 1c RESULTS, part 2**

1.PILOT STUDY: OPTIMIZATION OF ADOPTIVE THERAPY PROCEDURE. To establish a protocol, variable numbers of ErbB2-specific T cells were infused i.v. into tumor-bearing mice. The cells began to produce an effect -- significantly reduced tumor growth -- at 2 X 10^7 per infusion (Fig. 1). Maximal effect was achieved with 3 X 10^7 (7) and did not increase significantly when 4 or 5 X 10^7 cells were administered. Even with maximal effect tumors were not eradicated or permanently arrested. Relative to controls, tumors size was 30% at 7 days, 50% at 14 days, and not significantly different at 21 and 28 days.

The results of the pilot study show that infusions of 3 X 10^7 ErbB2-specific T cells produce a significant but incomplete and temporary reduction in the development of s.c. D2F2/E2 tumors. This is an ideal situation for determining whether cytoskeletal alteration of the T cells produces greater or longer-lasting improvement in immunotherapeutic effect.
Fig. 1. Effect of T Cell Infusion Size on Growth of D2F2 mammary tumor. Mice bearing bilateral ErbB2-expressing D2F2/E2 mammary tumors were given a single tail-vein infusion of anti-ErbB2 T cells stimulated in vitro. Infusions were given on day 14 after tumor implantation, which corresponds to day zero post-infusion of the X axis. Numbers of cells per infusion are given in the legend.

2. IMMUNOTHERAPEUTIC EFFECTS OF INCREASED TUMOR LOCALIZATION OF ML-7-TREATED T CELLS. As expected from the pilot study, a single infusion of T cells pretreated only with DMSO vehicle significantly reduced the growth of s.c. D2F2/E2 tumors for the first two weeks after treatment, relative to infusion of HBSS only (CONTROL vs. CELLS + VEH in Fig. 2). Also consistent with the pilot study, cell infusion had no significant effect on longer-term outcome. By 4 weeks post treatment all tumors had grown to a size that required euthanasia of host mice in both CONTROL and CELLS + VEH conditions (Fig. 2).

Surprisingly, despite their greater tumor localization, the ML-7-pretreated cells brought about therapeutic effects indistinguishable from those of vehicle-treated T cells (CELLS + ML-7 in Fig 2). Tumors in these mice showed the same temporary delay in growth and eventual relapse as those of mice infused with vehicle-treated T cells.

There are several possible explanations for the lack of effect. One is that is that the increased localization was offset by a long lasting depression of T cell function. We have shown that both proliferative activity and cytotoxicity quickly returns to normal levels in T cell populations given the same transient ML-7 treatment used in this study (Task 2a,b, below). It is still possible that cytokine secretion did not recover and that it may be necessary for antitumor effect in this system. Assays of cytokine secretion will be performed in the extension period of this project (Task 2c). Migration of activated T cells into extracellular matrix also recovered quickly after ML-7 treatment, but it is possible that the treated cells were impaired in their ability to extravasate into tissue in vitro. This possibility will be tested through analysis of the position of labeled cells in frozen sections of tumors from treated mice. The frozen blocks were obtained during the therapy study, and will be analyzed during the extension period. Finally, it is possible that even the fourfold increase in tumor localization produced by ML-7 pretreatment is not enough to make a difference in therapeutic effect. Even with ML-7-mediated
increase, only about 1% of the total cellular input reached the tumors. It is therefore important to continue to develop new and better strategies for improving the survival and tumor-homing of adoptively transferred T cells. Also worthy of consideration in future work is the possibility that the modest increase in localization attainable by ML-7 treatment may be therapeutically important when lymphocytes are used as vehicles of gene therapy rather than direct killers of tumor cells [8].

![Graph showing tumor diameter over time](image)

**Fig. 2.** Effects of ML-7-mediated transient depolarization of ErbB2-specific T cells upon their immunotherapeutic effect vs. D2F2 mammary tumor. Mice bearing bilateral ErbB2-expressing D2F2/E2 mammary tumors were given a single tail-vein infusion of anti-ErbB2 T cells stimulated in vitro. Infusions were given on day 14 after tumor implantation, which corresponds to day zero post-infusion of the X axis. Control, HBSS infusion only. Cells + Veh, T cells treated with DMSO vehicle 30 min, washed 3X, infused into tail vein in 0.5 ml HBSS. Cells + ML-7, T cells treated with 75 uM ML-7, washed 3X, and infused in HBSS. Values represent mean of a total of 6 mice (12 tumors) per condition.

**Task 1D.** Use fluorescent cell-tracking techniques to determine effects of myosin inhibiting drugs on localization of T cells in mice bearing experimental pulmonary metastases This work was planned for Year 2 of the project. All immunological studies in our animal facility were delayed for several months because of an outbreak of virus. This task will be performed during the no-cost extension period.

**Aim 2** To determine the effects of transient depolarization on immunotherapeutically important properties of antigen-specific T cells, including proliferative, cytolytic, and secretory response. This was to be accomplished in

**Task 2.** "Determine effects of myosin-inhibiting drugs on immunotherapeutically important functions of activated T cells in vitro"

**TASK 2A.** Determine effects of myosin-inhibiting drugs on proliferation in response to antigen stimulation.
TASK 2B. Determine effects of myosin-inhibiting drugs on specific cytotoxicity.

These first two subsidiary tasks were completed during the first year of the project and summarized in the last annual report. The findings were also alluded to in the interpretation of the therapeutic experiment, above. Briefly, when ErbB2-specific T cells were treated with ML-7 and washed, they recovered both normal proliferative potential and specific cell-mediated cytotoxicity against D2F2 target cells within 24 hr.

TASK 2C. Determine effects of myosin-inhibiting drugs on lymphokine secretion in response to antigen stimulation.

This will be completed during the no-cost extension period.

KEY RESEARCH ACCOMPLISHMENTS:

- Short-term inhibition of myosin function was found to reduce the trapping of adoptively transferred T cells in the pulmonary vasculature and to increase their localization in a murine mammary tumor and in lymph nodes.

- Cytotoxic function and proliferative capability of activated T cells quickly recover from the depressive effects of myosin function inhibition.

- These findings validate our hypothesis that the homing of immunotherapeutic T cells to tumors can be improved through manipulation of the T cell cytoskeleton.

- The results of our first immunotherapy experiment indicate that the improved homing we initially achieved is not sufficient to affect overall therapeutic outcome. Further refinement of cytoskeletal manipulation strategies is called for.

REPORTABLE OUTCOMES:

The products of the first year of work were reported at the Era of Hope Meeting, Orlando FL, September 2002 (poster P22-19). With the addition of results of Tasks 1c, 1d, and 2c, the work will be ready to submit for publication as the manuscript: "Increased tumor localization and diminished pulmonary trapping of adoptively transferred T cells after transient depolarization by inhibitor of myosin function."

CONCLUSIONS:

SUMMARY. The results of the in vivo localization experiments of Task 1 indicate that transient depolarization of activated T cells by a myosin light-chain kinase inhibitor allowed those cells to avoid massive trapping and damage in the pulmonary vasculature after i.v. infusion. The increase in surviving cells apparently led to a significant, fourfold increase in the percentage of infused cells to reach mammary tumors. There was also
greater homing to peripheral lymph nodes, where the T cells may recruit additional effectors and establish long term anti-tumor memory. However, in an immunotherapeutic trial, the improved homing did not benefit the outcome of an adoptive immunotherapy regime in mammary-tumor bearing mice. The results of Task 2 experiments performed so far demonstrate that the degree of myosin inhibition sufficient to alter T cell traffic causes only a brief decline in cytotoxic activity and ability to proliferate in response to antigen. The possibility is still open that myosin inhibition caused a longer-lasting depression of T cell cytokine secretion, a possibility still to be examined.

IMPORTANCE. The results constitute the first validation of the concept that direct alterations of the T cell cytoskeleton can improve the trafficking of therapeutic lymphocytes to sites of tumor growth. In this case, the manipulation was a simple transient pharmacological inhibition of function of the motor protein myosin, which is necessary both for the formation of cellular appendages and the maintenance of many types of adhesion receptor in activated configuration. In the future, more specific and controllable cytoskeletal alteration strategies may be developed for pre-infusion treatment of patient T cells. Among the likely possibilities would be antisense RNA against the members of the Rho family of GTPases, which regulate specific features of cellular polarity and motility [9,10].

"SO WHAT?" Adoptive immunotherapy is in theory an ideal way to provide at least short-term immunosurveillance to breast cancer patients whose immune systems have been debilitated by intensive therapy, but in practice the strategy is little used because of its poor track record in clinical trials. Our findings suggest a way to make adoptive immunotherapy more efficient and may therefore lead to a reconsideration of this mode of treatment.

The findings also have implications for the field of gene therapy. Lymphocytes are being considered as a possible vehicle for carrying genes and gene products into tumors. This strategy too could benefit from a method to make lymphocyte home more effectively to tumors [8].

CHANGES IN FUTURE WORK. None except, as mentioned above, final tasks will be completed during the no-cost extension period.

REFERENCES:


