Adoptive Immunotherapy by Avidin-driven Cytotoxic T Lymphocyte-Tumor Bridging

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ABSTRACT

We have shown previously that T cells, tagged with biotinylated anti-CD3 antibody fragments, can exert avidin-dependent cytolytic activity on suitably biotinylated tumor cells in vitro. In this study, we demonstrate that avidin-driven CTL-tumor bridging in vivo leads to growth inhibition of murine tumors WEHI-164 fibrosarcoma and RMA lymphoma. The biodistribution of biotin-tagged 111In-labeled T cells demonstrated a selective avidin-dependent and time-dependent accumulation of radioactivity at tumor sites. The specificity of lymphocyte tumor localization was demonstrated by the concurrent time-dependent decrease of radioactivity in the blood and in all other organs. Furthermore, we documented a therapeutic effect of the adoptively transferred T cells, i.e., a significant delay of tumor growth at early stages. All of the experiments included a control group of mice, which received all of the reagents, except avidin. These avidin-minus mice showed no specific localization and no delay in tumor growth, indicating that avidin bridging was essential for T-cell activity at tumor sites.

INTRODUCTION

The normal immune system can react with both humoral and cellular effector arms against malignant cells but rarely represents a significant barrier to tumor growth:

(a) A panoply of mAb1-defined tumor-associated antigens (B-TAA) is available, but the role of antitumoral antibody response in immune surveillance is controversial (1).

(b) A substantial number of T-TAAs, recognized by either CD8+ or CD4+ T cells, have been identified (2). Some of these antigens derive from tumor-specific mutations and some are unmodified shared antigens inappropriately expressed or overexpressed in transformed cells (3). Although it has been demonstrated, both in vitro and in vivo, that some T-TAAs can function as tumor rejection antigens (4), a poor correlation between induction of specific T cells and clinical responses has been observed (5).

(c) The loss of the MHC restricting element or the transporter associated with antigen presentation, which can occur in tumors (6–8), can be responsible for the relative inefficiency of T-TAAs as targets for cancer vaccines or immunotherapy. Poor T-TAA presentation can also depend on cytokine action; tumor cell lines expressing IL-10 strongly down-regulate MHC and the transporter associated with antigen presentation (9).

The advantages of antibody-based targeting and cell-mediated cytolyis have been combined in bispecific antibodies (10), which mediate tumor cell destruction by bridging a B-TAA on the surface of the tumor cell to a trigger molecule on the effector cell. Major efforts have been focused on TCR/CD3 molecules as trigger elements, sometimes combined with activation enhancers, i.e., costimulatory molecules or cytokines (11–13).

Exploiting a similar strategy, we have demonstrated recently that avidin-driven CTL-tumor bridging leads to T cell-mediated, non-MHC restricted cytotoxic activity on tumor cell lines in vitro (14). Lymphocytes were biotin-tagged by monovalent anti-CD3 antibodies, and tumor cells were pretargeted by biotinylated anti-B-TAA antibodies and avidin. In this report, we show that the same strategy applied in vivo leads to specific accumulation of biotin-tagged lymphocytes at pretargeted tumor sites in a non-MHC-restricted mode and to significant inhibition of tumor growth.

MATERIALS AND METHODS

Animals, Cell Lines, and Antibodies. C57BL/6 and BALB/c mice (females 4 weeks of age) were purchased from Charles River Laboratories (Calco, Italy). All mice were bred under pathogen-free conditions. As experimental tumor models, the Thy 1.1 transfectants of the following cell lines were used: the H-2b T-cell lymphoma RMA-1 (15), a Rauscher MuLV-induced tumor of C57BL/6 origin, and the H-2b fibrosarcoma WEHI 164 (ATCC # CRL-1751), a methylcolanthrene-induced tumor of BALB/c origin. Thy1.1-expressing cell lines (RMA-T and WEHI-T) were obtained by transfection with a construct encoding the Thy1.1 allele, inserted into the mammalian expression vector pRS1-neo (16). Cells were maintained in RPMI 1640 supplemented with 50 units/ml penicillin-streptomycin, 2 mM 1-glutamine, 50 μg/ml mercaptoethanol, 10% heat-inactivated FCS, and 500 μg/ml G418 (Sigma Chemical Co., St. Louis, MO). mAbs 19E12 (anti-Thy1.1; kindly supplied by Dr. A. Livingstone, Basel Institute for Immunology, Basel, Switzerland) and 2C11 (anti-CD3 epsilon; Ref. 17) were obtained either from culture supernatants of hybridoma cells or from ascitic fluids. F(ab′)2 fragments of mAb 2C11 were obtained by papain digestion, according to standard protocols, mAb purity was evaluated using SDS-PAGE analysis. F(ab′)1 fragment biotinylation was performed as follows. One ml of 0.5 mg/ml antibody solution in sodium bicarbonate buffer (pH 8.8) was mixed with 100 μl of 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce Chemical Co., Rockford, IL) solution. After 4 h incubation at room temperature, 125 μl of 1 M lysine solution were added for 2 additional h. Biotinylated mAbs were dialyzed overnight against PBS.

Flow Cytometry. Binding and persistence of biotinylated 19E12 on tumor cells and of biotinylated 2C11 F(ab′)2 fragments on T cells were evaluated by fluorescence-activated cell sorter analysis. Cells were incubated with 10 μg/ml biotinylated antibodies in PBS, containing 3% FCS, for 1 h on ice. The cells were washed in medium and incubated for 1–24 h and washed and stained with fluorescein-conjugated streptavidin. Mean fluorescence intensity was used to estimate the amount of antibodies bound to the cells at each time point.

Preparation of Biotin-tagged T Cells for Adoptive Transfer. Activated T cells were obtained from splenocytes isolated from C57BL/6 or BALB/C mice and cultured on anti-CD3-coated 24-well plates in RPMI 1640 supplemented with antibiotics and 10% FCS. After 3 days, recombiant IL-2 (100 units/ml) was added, and T cells were expanded in vitro for 3 additional days. Cells were then collected and incubated with biotinylated 2C11 F(ab′)2 fragments (1 μg/106 cells) for 1 h at 4°C. After extensive washing, biotin-tagged cells were suspended in sterile 0.9% NaCl before in vivo adoptive transfer experiments.

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**In Vivo Studies.** Studies on animal models were approved by the Ethical Committee of the San Raffaele Scientific Institute and performed according to the prescribed guidelines. C57BL/6 and BALB/c mice were inoculated, respectively, with $5 \times 10^3$ RMA-T or $7 \times 10^4$ WEHI-T cells, in the right flank. At various days after tumor implantation (days 1–4, as indicated for each of the reported experiments), mice were treated with sequential administrations of biotinylated antibodies, avidins, and biotin-tagged T cells, according to the following 3-day protocol: step 1, $40 \mu$g of biotin-mAb 19E12 (i.p.); step 2, after 18 and 19 h, respectively, $60 \mu$g of avidin and $60 \mu$g of streptavidin (i.p.); and step 3, 24 h later, $5-20 \times 10^8$ biotin-tagged lymphocytes (i.v.). Steps 1 and 2 are collectively called “pretargeting steps,” whereas step 3 is called “adoptive transfer.” Each reagent was diluted in sterile 0.9% NaCl. In control experiments, one or more of the above components were replaced with diluent. Where indicated, 2000 units IL-2 were administered i.p. to each mouse for 3 days, starting from the day after adoptive transfer. Each experiment was carried out with five mice/group. Treated mice were monitored every second day for tumor growth by measuring two perpendicular diameters with a caliper. Tumor volume was estimated by calculating $V = (d_s \times d_l \times d_l)/2$, where $d_s$ and $d_l$ are the short and long diameters, respectively. Animals were killed when the tumors became larger than 500–600 cubic mm.

**Biodistribution of $^{111}$In-labeled Lymphocytes.** Activated biotinylated lymphocytes ($\sim 10^8$) were labeled with 29–37 MBq of $^{111}$In-Oxine (Mallinckrodt Medical, Petten, Holland), according to reported methods (18), obtaining a specific activity of 1.0–1.3 MBq/$5 \times 10^8$ lymphocytes. After the completion of the pretargeting steps on day 5, the adoptive transfer was performed on day 6 by i.v. injection of $5 \times 10^8$ biotin-tagged $^{111}$In-labeled lymphocytes into BALB/c mice implanted with WEHI-T tumors. After 4 and 24 h from the adoptive transfer, five mice/group were euthanized, and spleen, liver, kidney, lung, bone marrow, blood, urine, and tumor were collected and analyzed for the associated radioactivity in a gamma counter. The results are represented as the mean $\pm$ SD of percentage of total dose injected/ingesting of tissue.

**Statistical Analysis.** Tumor sizes are shown as the mean $\pm$ SE. Statistical analyses on tumor growth data were performed using covariance analysis. Statistical analyses on survival data were performed using the log rank test.

**RESULTS**

**Adoptive Transfer of Biotin-tagged Lymphocytes.** Avidin bridging of biotinylated lymphocytes and tumor cells in vitro elicits antibody-driven, T cell-mediated, non-MHC-restricted cytotoxic activity (14). To verify the immunotherapeutic potential of this strategy in vivo, we applied it to two mouse tumor models (BALB/c WEHI-T and C57BL/6 RMA-T). Because both mouse strains have a Thy 1.2 genotype, the cell lines were transfected with the Thy 1.1 gene to mimic a unique B-TAA-equivalent, reactive with mAb 19E12 (16).

Lymphocytes from the spleens of C57BL/6 or BALB/c mice were activated and expanded in vitro by exposure to solid-phase mAb 2C11 (anti-CD3e) and to IL-2 (see “Materials and Methods”). After 6 days in culture, T lymphocytes were biotinylated via F(ab')$_2$ proteolytic fragments of the mAb 2C11, which, in the soluble form, do not mediate T-cell activation (not shown).

The persistence of these lymphocytes of biotinylated 2C11 F(ab')$_2$ was measured by flow cytometry. After an initial 30% loss in the first hour, the half-life of the surface anti-CD3 F(ab')$_2$ was about 8 h (Fig. 1). Conversely, the anti-Thy 1.1 mAb persisted on RMA-T and WEHI-T tumor cells without detectable decrease for at least 24 h (not shown). Biotin-tagged lymphocytes were adoptively transferred into tumor-bearing mice treated with the pretargeting steps (see “Materials and Methods”).

**Tumor Localization.** The effects of tumor pretargeting and lymphocyte tagging on lymphocyte biodistribution were evaluated by tumor localization experiments in the BALB/c WHEI-T model, using biotin-tagged $^{111}$In-labeled lymphocytes. The controls were tumor-bearing mice in which one or both pretargeting reagents had been omitted. Animals were sacrificed 4 or 24 h after the adoptive transfer. As shown in Fig. 2, after 4 h, lymphocyte-associated radioactivity could be detected in all compartments, with higher concentrations in lung, kidney, spleen, and liver, and a tumor: blood ratio of 0.5. At 24 h, a selective accumulation of radioactivity in the tumor compartment was observed, and the tumor: blood ratio increased from 0.5 to $\sim$10.

The tumor was the only compartment with a time-dependent increase in radioactivity (>2-fold), whereas all other compartments showed a time-dependent decrease. The kinetic pattern shown by the tumor compartment was significantly different ($P < 0.01$ to $P < 0.001$) from those of all other compartments. Avidin-minus controls showed significantly lower counts ($P < 0.001$) only in the tumor compartment.

These data indicated that biotin-tagged lymphocytes accumulate and are specifically retained by pretargeted tumors via avidin bridging. Statistical analysis demonstrated that the compartments (other than tumor) could be grouped into two groups; blood, urine and lung showed a marked time-dependent decrease in radioactivity, whereas kidney, bone marrow, liver, and spleen showed a less marked decrease. The difference between the two patterns was statistically significant ($P < 0.001$).

**Tumor Therapy.** In the BALB/c WEHI-T model, the adoptive transfer of biotin-tagged lymphocytes at day 5 blocked tumor growth at the volume reached at day 7 in control mice (Fig. 3). The difference in tumor growth was statistically significant ($P < 0.001$). The addition to the protocol of three administrations of low-dose rIL-2 (2000 units i.p./mouse at days 6, 7, and 8) further enhanced the antitumor effect, resulting in a block of tumor growth at the size reached at day 6 in control mice, possibly because of a facilitating effect on lymphocyte extravasation. The cytokine treatment had no effect on tumor growth in a control group. The experiments with WEHI tumors can give information only on the early stages of tumor growth, because a spontaneous rejection of the tumor takes place at days 12–13.

Conversely, in the C57BL/6 RMA-T model, tumors are very aggressive and result in death by days 15–20. Also in this model, a highly significant delay in tumor growth ($P < 0.001$) was observed in animals adoptively transferred with biotin-tagged lymphocytes and treated with low-dose rIL-2, whereas incomplete control therapies, lacking one of the steps, did not show any antitumor effect (Fig. 4A and data not shown). The antitumor effect was, in this model, also documented by a marked prolongation of survival (i.e., the time to reach the dimensional end point at which animals have to be sacrificed). In the experiments reported in Fig. 4B, 9 of 10 control animals had to be sacrificed before the first treated animal reached the end point, the difference between the survival curves being highly significant ($P < 0.001$).

In one experiment, where the adoptive transfer of biotin-tagged lymphocytes was performed earlier (completed at day 3), no antitumor effect could be observed (not shown), consistent with the notion that...
tumor vascularization starts around day 5 and that, at that date, the concentration of biotinylated 2C11 F(ab')2 fragments on the surface of lymphocytes might have become too low to mediate homing to the tumor.

**DISCUSSION**

The safety and the clinical proficiency of a pretargeting “three-step method” based on avidin-biotin bridging to deliver radioisotopes selectively to tumors have been demonstrated conclusively (19–21). The method consists of the following steps: biotinylated anti-B-TAA monoclonal antibodies (first step) and, 1 day later, avidin (second step) are administered to the patient; a biotin-tagged chelate-radioisotope complex (third step) is then administered after a suitable interval, when avidin and avidin-biotinylated antibody complexes have been removed from the circulation (22–24). The biotin-tagged radioisotope is then selectively retained by the avidin bound on the tumor cell surface or rapidly lost through the kidney, reaching tumor:background ratios of 20–100. Both diagnostic and therapeutic applications have been described and introduced into clinical practice (21–23).

Because of its tetravalency, tumor-bound avidin is potentially able to function as an “artificial receptor” for a variety of effectors, provided that they are biotinylated. The selectivity is guaranteed by the very efficient liver-mediated clearance of circulating avidin and avidin-biotin complexes, which ensures that, just 4 hours after avidin administration, the only accessible avidin in the organism is that which is bound to the tumor. The artificial avidin receptor has been successfully used to selectively direct biotinylated TNF-α onto tumors (24).

The aim of the present work was to exploit the artificial avidin receptor for redirecting biotinylated autologous cytotoxic cells onto tumors (in a non-TCR-specific, non-MHC-restricted mode) to obtain selective, avidin-driven, in situ activation of cytotoxic potentials. The selective biotinylation of a trigger molecule such as CD3 (achieved by treating the cells with biotinylated anti-CD3 antibody fragments) drives the selective killing of avidinated tumor targets in vitro; conversely, chemically biotinylated lymphocytes, although fully viable, fail to kill avidinated tumor targets (14).

CD3 aggregation, and therefore activation of the cytotoxic machinery, must occur only on the tumor surface, mediated by avidin bridging. Should such cross-linking occur before, TCR internalization would remove the biotinylated ligand from the cell surface, thus interfering with redirected tumor homing. In our preliminary in vitro work with human lymphocytes and cell lines, we used monovalent...
Avidin-driven CTL-tumor bridging in vivo

Fig. 4. A, delay of tumor growth in the C57BL/6 RMA-T tumor model, attributable to the adoptive transfer of $5 \times 10^6$ (•) or $2 \times 10^7$ (○) biotin-tagged lymphocytes into mice bearing pretargeted tumors, as compared with the adoptive transfer of the same numbers of lymphocytes into avidin-minus control mice (open symbols). The pretargeting steps were completed on day 5 from tumor implantation, and the adoptive transfer was performed on day 6. All mice also received three administrations of rIL-2 (2000 units i.p./mouse at days 6, 7, and 8). B, difference in survival (i.e., the time to reach the dimensional end point at which animals have to be sacrificed) between the pooled treated animals (○) and the pooled avidin-minus controls (□).

scFv reagents, but these have a limited avidity and thus a short half-life (~4 h) on the cell surface, and it is unlikely that they could ever be used in a clinical setting. In mouse experimental systems, anti-CD3 F(ab')2 fragments do not induce T-cell activation, and as shown in Fig. 1, have a longer half-life on the cell surface (~8 h), more likely to be enough for localization purposes.

Biotin-tagged radioactive lymphocytes showed a background biodistribution in avidin-minus control mice qualitatively and quantitatively analogous to that reported for adoptively transferred $^{111}$In-labeled tumor-infiltrating lymphocytes in metastatic melanoma patients (25). The effect of avidin was dramatic; the tumor:blood ratio, which was 0.5 at 4 h, was inverted and grew to about 10 at 24 h, the tumor being the only compartment with a time-dependent increase in radioactivity, while all other compartments showed a time-dependent decrease.

In essence, the results demonstrate that: (a) avidin can efficiently drive homing of biotin-tagged lymphocytes to pretargeted tumor sites; and (b) redirected lymphocytes display antitumor activity in two mouse tumor models in vivo. We have therefore reached the same goals sought by bifunctional antibody strategies. However, the availability of reagents, the ease of biotinylation, the complete and reliable avidin-driven clearance of biotinylated antibodies, the high affinity of avidin-biotin interaction, and the possibility of using antibody combinations (to avoid the escape of antigen-negative tumor variants) are clear advantages of this approach, as compared with bispecific antibodies. Moreover, separating lymphocyte tagging from tumor pretargeting allows optimization of each step independently from the other, thus allowing construction, on a given tumor, of an “optimal” artificial receptor, which could even be reliably monitored with a biotinylated radioisotope in tracer amounts.

An appealing evolution of this strategy could be to use genetically modified T cells expressing a chimeric TCR composed of an avidin-reactive extracellular moiety and of the signal transducing sequence of the ζ chain of the TCR/CD3 complex. CTLs expressing antigen-reactive chimeric receptors (T-bodies) have been shown to have cytotoxicity against tumors both in vitro and in vivo (26–29). The major drawback of T-bodies was the inhibition of chimeric receptor activity by soluble antigen, thus limiting their application only to integral membrane protein target antigens (29). This limitation should not exist for avidin-reactive CTLs, because avidin would be completely cleared from the circulation at the time of adoptive transfer.

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