Immunotherapy of Human Tumors with T-Cell-activating Bispecific Antibodies: Stimulation of Cytotoxic Pathways in Vivo

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ABSTRACT

Bispecific monoclonal antibodies (Bi-mAbs) specific for a tumor-associated antigen and the CD3 or CD28 antigen on T lymphocytes represent one of the most successful experimental strategies for the immunotherapy of cancer. We report that the in vivo administration of both α-CD3/CD30 and α-CD28/CD30 Bi-mAbs results in the specific activation of xenotransplanted, resting human T cells infiltrating the CD30-positive Hodgkin’s tumor. Bi-mAb treatment resulted in enhanced expression of cytokines such as interleukin 1β, interleukin 2, tumor necrosis factor type α, and activation markers including Ki-67, CD25, and CD45RO in tumor-infiltrating lymphocytes. This antigen-dependent, local T-cell stimulation led to the activation of the cytolytic machinery in T lymphocytes, determined by the up-regulation of mRNA-encoding perforin and the cytotoxic serine-esterases granzymes A and B. The Bi-mAb-induced generation of CTLs depended on the presence of the CD30 antigen and the combined application of both Bi-mAbs. Our findings suggest that the combined application of T-cell-activating Bi-mAbs is able to achieve a tumor site-specific activation of the T-cell cytolytic machinery in vivo. The fact that these cytotoxic cells do not home in tumor-associated antigen-negative tissue and do not enter circulation might explain our previous observations (C. Renner et al., Blood, 87: 2930–2937, 1996) of a high cure rate in preclinical models even at an advanced stage of disease.

INTRODUCTION

T cells are commonly recognized as the most potent effector cells to generate antitumor cytotoxicity (1). Many approaches of experimental cancer treatment try, therefore, to induce T-cell immunological effector mechanisms leading to tumor eradication (2, 3). One of the most successful strategies is represented by Bi-mAbs,3 which mimic the physiological T-cell stimulation initiated originally by an antigen-presenting cell. Bi-mAbs that target with one arm a TAA and with the other arm a T cell triggering molecule are able to activate specifically resting T cells, which results in tumor destruction (4, 5). According to the current concept of T-cell activation (6), a combination of two Bi-mAbs that recognize with one site a TAA and with the other the CD3 or CD28 antigen on T cells, respectively, are needed for an antigen-specific-but-MHC-unrestricted T-cell activation (7, 8). Using Hodgkin’s lymphoma as a model, we demonstrated that the bridging of Hodgkin’s tumor’s cells to T-cell triggering molecules by a combined α-CD30/CD3 and α-CD30/CD28 Bi-mAb regimen induced an efficient tumor cell lysis in vitro and in vivo (9). In a preclinical model, the treatment of SCID mice harboring xenografted disseminated CD30+ tumors resulted in the complete cure of all of the animals. Even if the beginning of treatment was delayed and tumor growth advanced, complete remissions could be achieved (10). Our data confirmed previous observations (11) that only the combination of both CD4+ and CD8+ lymphocytes resulted in the cure of tumor-bearing SCID mice, which suggested that both subsets are crucial for the efficient eradication of tumor cells (10).

However, the precise way by which Bi-mAb-activated T cells eliminate tumor cells still has to be elucidated. Early experiments with CTLs stressed the role of cytokines in tumor cell lysis (12). These results were recently challenged by experimental evidence that the contribution of cytokines to tumor cell lysis is not by direct participation in processes involved in rapid cell destruction (13); rather, cytokines are now believed to function primarily by down-regulating the cell-cycle activity of tumor cells and by augmenting the cytolytic activity of effector lymphocytes (14). The key components in the cytolytic machinery of T cells are represented by perforin and granzymes; as potent cytotoxic molecules they can suppress tumor growth (15). After attaching at the target cells, perforin monomers assemble to polymeric pore structures, which are inserted into the plasma membrane thus causing osmotic cytolysis and the typical picture of necrosis (16). Secreted granzymes enter the target cell using the perforin-channel and initiate an internal disintegration pathway that leads to DNA fragmentation (apoptosis) and lysis (17). In vitro data showed that this granule-exocytosis pathway plays a central role in bispecific-antibody-induced T-cell-mediated tumor destruction (18). In accordance with the observation that CD8+ CD45RO+ cells are the most efficient cytotoxic cells, the up-regulation of expression of perforin and granzymes A and B reached the highest levels in this cell population (19). To investigate whether these molecular programs of Bi-mAb-induced cytotoxic T cells are also operative in the immune destruction of tumor cells in vivo, we established an experimental tumor model in C.B-17 SCID/SCID mice. Simultaneous growth of either CD30-positive Hodgkin’s lymphoma and TAA-unrelated colon carcinoma was induced s.c. Our investigations show that human lymphocytes activated by the combination of both Bi-mAbs are found only in Hodgkin’s lymphoma tissue and to a much lesser extent or not at all in TAA-negative tissue including the unrelated colon carcinoma. This site-specific activation of resting human T lymphocytes led to directed exocytosis of perforin and granzymes, demonstrating that this pathway is a very important mechanism for Bi-mAb-mediated tumor destruction in vivo and the consecutive cure of animals xenografted with disseminated Hodgkin’s lymphoma, even in advanced stages of disease.

MATERIALS AND METHODS

Mice. Pathogen-free, 4–6-week-old mice with SCID (C.B-17 lcr SCID/SCID) were obtained from the Institut für Versuchstierzucht (Hannover, Germany). Animals were housed and bred in laminar flow racks and fed with autoclaved standard chow and water. “Leaky” animals were identified by ELISA (20) and excluded from further treatment. For in vivo-depletion of murine natural killer cells, all of the mice used in the experiments were pretreated with 100 μl of anti-asialo-GM1-antibody (Wako, Japan) i.p. as described previously (21).

Antibodies and Cell Lines. The generation, purification, and characterization of the Bi-mAb (α-CD3/CD30 and α-CD28/CD30) have been described

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3 The abbreviations used are: Bi-mAb, bispecific monoclonal antibody; TAA, tumor-associated antigen; SCID, severe combined immunodeficient; mAb, monoclonal antibody; TNF, tumor necrosis factor; IL, interleukin; FAC/S, fluorescence-activated cell sorting; RT, reverse transcription; TIL, tumor-infiltrating lymphocyte.

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on a thermocycler (MWG-Biotech, Ebersberg, Germany) with the following profile: 95°C for 80 s, annealing for 80 s, and extension at 72°C for 2 min. The number of cycles and the annealing temperature depended on the primers used: (a) perforin (57°C, 30 cycles); (b) granzyme A (50°C, 32 cycles); (c) granzyme B (61°C, 30 cycles); and (d) β-actin (61°C, 30 cycles). Aliquots (20 μl) of each PCR product were separated on a 2.5% agarose gel and visualized by ethidium bromide staining (Sigma).

**Northern Blot Analysis.** Northern blots were performed as described previously (19). 5′-32P-CTP-random-labeled (Ready-To-Go Kit, Pharmacia, Freiburg, Germany) gene-specific cDNA probes were hybridized in a formamide buffer system (5 ml of formamide, 2.5 ml of 20 × SSC, 1 ml of 50 × Denhardt’s solution, 0.5 ml of SDS 20%, 1 ml of double-density H2O, and 50 μg/ml heat-denatured salmon sperm) at 42°C for 10–16 h. After hybridization, membranes were washed and exposed at −70°C. The perforin and granzymes A- and B-specific cDNAs were generated by PCR as described previously (18).

**RESULTS**

**Bi-mAb-induced Secretion of T-Cell Cytokines and Expression of Activation Markers in Vivo.** CD28-costimulation of TCR/CD3-activated T lymphocytes can augment cell proliferation and production of multiple cytokines (e.g., IL-2 and TNF-α) and enhance expression of the high-affinity receptor for IL-2 (CD25; Ref. 28). As demonstrated by FACScan-analysis (Fig. 1), the same effect can be achieved by the combined application of our Bi-mAbs, cross-linking the CD3 and CD28 antigen on resting T cells in the presence of CD30+ tumor cells. The highest number of proliferating T cells expressing the cell nucleus-associated antigen Ki-67 as a marker for human cells entering the cell cycle was measured in the CD30+ positive Hodgkin’s lymphoma when activated appropriately by both Bi-mAbs (HT3 and 2F10). The majority of stimulated lymphocytes remained in the TAA-positive tissue and did not re-enter into circulation. Neither the TAA-negative tissue nor the unrelated colon carcinoma showed relevant counts of anti-CD3/-anti-Ki-67 double-positive stained cells. CD3+ lymphocytes from those animals treated with one Bi-mAb alone (HT3 or 2F10, respectively) or from the control-group rested in the G0 phase of the cell cycle. Proliferating T cells from the group of mice with combined Bi-mAb treatment showed 2- to 4-fold higher peak levels of IL-1β, IL-2, and TNF-α when compared with all of the other treatment-groups. Cross-reactivity of the anti-IL-2-moAb between murine and human IL-2 was excluded by using double immunofluorescence. The high expression level of the CD25 antigen on TILs after combined Bi-mAb treatment confirmed their activation status.

The in situ activation of the resting human T lymphocytes via cross-linking of both CD3/CD30 and CD28/CD30 Bi-mAbs by solid-growing CD30+Hodgkin’s lymphoma led to the expansion of the memory lymphocyte pool characterized by the expression of the CD45RO “memory” antigen. Lymphocytes expressing the CD8+CD45RO phenotype are the most potent mediators of target cell lysis in vitro and induce a significant higher rate of cytotoxicity when compared with naive CD8+ lymphocytes characterized by the CD45RA antigen (19). Lymphocytes infiltrating Hodgkin’s tumors were represented by both CD4+CD3+ and CD8+CD3+ T cells (Fig. 1B), which was significantly higher expressed by the CD8+ cells.

**Up-regulation of Perforin and Granzyme Message in Bi-mAb-activated T Cells in Vivo.** As perforin and the granzyme family seem to be the most important mediators involved in target cell lysis, we looked for the up-regulation of these molecules in Bi-mAb-activated tumor infiltrating lymphocytes by Northern blot and RT-PCR analysis. Perforin and granzyme A and B mRNA were expressed only in Hodgkin’s lymphoma of SCID-mice treated
with the Bi-mAb combination regimen (Fig. 2, animal 1, Lane 2) and was not detectable by Northern blot in the CD30-negative colon carcinoma (Lane 1) nor in the spleen (Lane 3) or blood (Lane 4). There was no induction of these cytotoxic proteins in animals treated with one Bi-mAb alone because detection of granzyme and perforin gene expression failed. Missing of cytotoxic activity after inappropriate treatment is represented by animal 2 (Fig. 2), which was treated with anti-CD3/CD30 Bi-mAb only. The differential pattern of mRNA expression for perforin and granzymes A and B appeared to be a consequence of the treatment regimen used because the expression of β-actin gene was similar in all of the tissues resected from different experimental groups.

Dependence of Bi-mAb-induced Cytotoxicity on the Expression of the CD30 Antigen in Vivo. In a second set, we performed a RT-PCR analysis (Fig. 3) to confirm the Northern blot results and to clarify that the Bi-mAb-mediated induction of T-cell cytotoxicity is strictly dependent on the targeted TAA. The different methods revealed adequate results; consistent induction of perforin and granzymes A and B mRNA was dependent on the presence of the CD30 antigen and the combined application of the anti-CD3/CD30 and anti-CD28/CD30 Bi-mAb. The key cytotoxic components were only detectable in the Hodgkin’s tumors of those mice treated with the combined Bi-mAb regimen (Lanes 1–4) and could not be detected in tissues from mice treated with Bi-mAb (Lanes 1–4).

Fig. 1. FACScan-analysis of cytokine-secretion and antigen-expression of xenografted resting human T lymphocytes after in vivo activation by combined application of α-CD3/CD30 and α-CD28/CD30 Bi-mAbs. Solid tumor-bearing mice were treated by i.v. injection of resting human T cells followed by Bi-mAb application. Animals were killed after 7 days, and tissues were removed to evaluate the in situ activation of T cells. A, cytokine levels or activation marker expression of anti-CD3-PE-pated T lymphocytes were measured by intra/extracellular staining with secondary FITC-conjugated mAbs. Columns, the percentage of cytokine/antigen positive cells within the pool of activated CD3+ T cells (mean ± SD; ten mice per treatment-group). Animals were treated with HT3+2F10 (□), HT3 (■), 2F10 (▲), or PBS (▲), and expression of indicated cytokines/proliferation markers were measured. The type of tumor and normal tissue analyzed is stated on the X axis. B, differential infiltration of established Hodgkin’s tumors with CD45RO+ memory cells depending on antibody treatment. Simultaneously growing Hodgkin’s and colon tumors were resected from mice treated with different antibody combinations (HT3+2F10 (□), HT3 (■), 2F10 (▲), or PBS (▲)), and tumor tissue was analyzed for infiltration with CD45RO+ cells. Within the CD45RO+ population, cells were further divided into CD8+ and CD4+ lymphocytes. For each antibody group, values obtained from colon tumors were subtracted from values obtained from Hodgkin’s tumors to correct for unspecific lymphocyte homing in antigen-negative tissue.
specific for perforin or granzyme A or B. A blood
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studies between mice treated with one Bi-mAb alone or a combination
ence in lymphocyte accumulation could be observed in our previous
reached significantly higher distribution values in tumor tissue when
vivo
in which Bi-mAb-activated lymphocytes induced tumor cell lysis
obtained earlier in vitro (22). Both CD4+ and CD8+ cells contributed
to the Bi-mAb mediated cytotoxicity, with the CD8+ lymphocytes
representing the most effective subset (22). However, the precise way
in which Bi-mAb-activated lymphocytes induced tumor cell lysis in vivo
still remained uncertain and had not yet been addressed. Several
studies dealing with this topic thus far proved that Bi-mAbs and
Bi-mAb-coated lymphocytes accumulated at the tumor site and
reached significantly higher distribution values in tumor tissue when
compared with antigen-negative tissue (9, 29). No significant differ-
ence in lymphocyte accumulation could be observed in our previous
studies between mice treated with one Bi-mAb alone or a combination
of both. We favor the hypothesis that the successful eradication of
human tumors was not due to quantitative differences in lymphocytes
targeted to the tumor site but was rather caused by in situ activation of
human T cells entering the tumor compartment. The present SCID
mouse model with solid Hodgkin’s and colon tumors established in
the same mouse at the same time confirmed this hypothesis inasmuch
as expression levels of cytokines that were measured in different
tissues revealed a strict dependence on the expression of the CD30
antigen. The concentration of IL-1β, IL-2, and TNF-α and the ex-
pression values for CD25 and Ki-67 were manifoldly higher in lymphocytes infiltrating Hodgkin’s lymphoma than in colon tumor tissue.
Moreover, we showed that the majority of activated T cells remained
in the CD30+ tissue and did not enter the circulation. Efficient
stimulation of CD3+ lymphocytes was induced only after injection of
both CD3/CD30 and CD28/CD30 Bi-mAbs. The crucial role of the second
signal for efficient T-cell activation in vivo has been empha-
sized recently by clinical data. Patients with B-cell lymphoma treated
with one anti-CD3/CD19 Bi-mAb alone had only slightly enhanced
levels of cytokines with no significant T-cell enrichment in tumor
tissue could be observed (30). However, the addition of IL-2 as
second signal initiated efficient T cell activation proven by the in-
creased expression of CD25 and the high levels of cytotoxic granules
in tumor infiltrating lymphocytes (31).
Analyzing the subsets of T cells infiltrating Hodgkin’s lymphoma
showed the highest levels for the CD8+ CD45RO+ pool. The phenomena
t that memory CD8+ T-cells are in vitro much more potent effector
cells than naive CD8+ T cells was first described by Azuma et al. for
CTL clones (32) and could be confirmed by our group for Bi-mAb-
mediated cytotoxicity (22). Moreover, Demanet et al. (4) demonstrated in
an in vivo murine lymphoma model that unprimed mice did not reject
tumors efficiently despite the fact that two T-cell activating antibodies
were used. However, if animals were primed before treatment with
allogeneic EL 4 lymphoma cells, a rapid expansion of T cells expressing
the CD45RO memory antigen could be detected leading to a high
remission rate after subsequent tumor challenge and treatment with two
Bi-mAbs, one recognizing the CD3 antigen and the other one the CD28
antigen on T cells. Therefore, memory T cells seem to have a crucial
impact on tumor cell elimination. In our model, the combined application
of Bi-mAbs initiating the primary and the costimulatory signal at the

![Fig. 2. Analysis of perforin and granzymes A and B mRNA expression in different tissues after antibody treatment. Total RNA for Northern blot analysis was isolated from resected tissues as described in “Materials and Methods.” Mice were treated with either a combination of both Bi-mAbs (animal 1) or just the HT3 Bi-mAb (animal 2). The following tissues were resected and analyzed: Hodgkin’s tumor (Lane 2), spleen (Lane 3), blood (Lane 4), or an unrelated colon carcinoma (Lane 1). Blots were probed with cDNAs specific for perforin or granzyme A or B. A β-actin-specific probe was used to ensure equivalent RNA load in all of the lanes.](Image 319x121 to 548x363)

![Fig. 3. RT-PCR analysis of perforin and granzymes A and B expression in different tissues after antibody treatment. RT-PCR for perforin, granzymes A and B, and β-actin was performed as described in “Materials and Methods.” Lane 1–4 represent Hodgkin’s tumors resected from four mice treated with a combination of both Bi-mAbs. DNA marker is shown in Lane 5 for all of the samples. Lane 6–9 represent cDNA generated from different tissues resected from mice treated with a combination of both Bi-mAbs. The order of the tissues analyzed is as follows: spleen (Lane 6); Hodgkin’s lymphoma (Lane 7); peripheral blood (Lane 8); and a colorectal carcinoma as CD30 antigen-negative tumor control (Lane 9).](Image 55x519 to 285x741)
tumor site ensured the TAA-dependent expansion of cytotoxic T cells not home to TAA-negative tissues, the extent of side effects and normal killing of tumor cells by activated T cells suffering from follicular lymphoma, TILs displayed high levels of perforin, which was interpreted as a marker of cytotoxic activity and was speculated to be caused by the endogenous local secretion of IL-2 by the tumor cells (35). A similar result was published for patients with metastatic melanoma. In these patients, s.c. administration of IL-2 enhanced the pool of circulating perforin- or granzyme-positive lymphocytes in a dose-dependent fashion and modulated tumor-specific cytotoxicity (36).

Our results underline the major advantage of the combined anti-CD3/anti-CD28 Bi-mAb approach by inducing a tumor site-specific activation of the T-cell cytolytic molecular program in vivo. The granule exocytosis model does not depend on receptor-ligand interactions and, in contrast to the FAS/APO-1 system, is not limited to FAS/APO-1-positive cells. Therefore, the described in vivo findings of Bi-mAb-mediated cytotoxicity should be transferable to a wide range of tumors irrespective of their FAS/APO-1 antigen status. As Bi-mAb-targeted- and -activated lymphocytes did not home to TAA-negative tissues, the extent of side effects (caused e.g. by damage of normal tissue by activated T cells) should be low.

REFERENCES

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