

Bispecific Antibodies Target Operationally Tumor-Specific Antigens in Two Leukemia Relapse Models

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Despite improved procedures in chemotherapy and bone marrow transplantation (BMT), post-BMT leukemia relapse rates have remained rather constant in the last decade. Immunotherapy with monoclonal or bispecific antibodies (bsAb) is a promising approach to improve this situation, but is hampered by the absence of tumor-specific antigens on the majority of tumors. To evade this problem, we developed a new tumor-specific approach in which bispecific antibodies exploit chimerism after allogeneic BMT by redirecting donor T cells against recipient-specific antigens on tumor cells. Two different leukemia relapse models were established using a T-cell lymphoma (ST-1) and a B-cell lymphoma (BCL1) to evaluate the efficiency of such a therapy. In these experiments, irradiated BALB/c (Thy-1.2⁺, I-A^d) mice were transplanted with C57BL/6 Thy-1.1 (I-A^b) BM cells under the protection of graft-versus-host disease-preventing monoclonal antibodies. Forty-five days after BMT, the chimeric mice were injected with either 2×10^4 recipient-type, Thy-1.2⁺, CD3⁻ ST-1 cells or major histocompatibility complex (MHC) class II⁺ (I-A^d)-BCL1 cells. Four days later, the mice were treated with 8 μ g bsAb G2 (anti-CD3 $\times$ anti-Thy-1.2)

or 10 μ g (+10 μ g, day 6) bsAb BiC (anti-CD3 $\times$ anti-I-A^d), respectively. These combinations guaranteed exclusive binding of the bsAbs target arms to tumor cells, leaving the surrounding, donor-type hematopoietic cells unbound. Compared with the parental antibodies, the bsAbs markedly reduced tumor mortality. Between 34% and 83% of mice survived in the bsAb groups compared with 0% of the control groups treated with parental antibodies, clearly documenting the benefit of the redirection principle. Furthermore, cytokine release (interleukin-6) after anti-CD3 antibody or bsAb treatment was decreased by administering a low-dose antibody preinjection. We have shown (1) that 6 weeks after BMT, when donor T-cell reconstitution is still in progress, T-cell-redirecting bsAb are clearly superior to parental antibodies in terms of tumor cell elimination; and (2) that the polymorphism of a common antigen such as Thy-1 or a clinically more relevant target antigen such as MHC class II can be used as an operational tumor-specific antigen after allogeneic BMT.

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THERAPEUTIC OPTIONS for patients with recurrent leukemia after bone marrow transplantation (BMT) are limited. A promising approach for such resistant leukemias is immunotherapy with, eg, donor lymphocyte infusions.^{1,2} Donor lymphocyte infusion can cure especially recurrent chronic myelogenous leukemia, probably by the action of T cells, but is complicated by the possibility of simultaneously induced graft-versus-host reaction. Therefore, a more specific and better controllable therapy, eg, with monoclonal antibodies (MoAbs) that target lymphocytes to residual leukemic cells, is desired. However, in most cases, unlabeled or unconjugated antibodies do not activate immune effector mechanisms sufficiently to eradicate all tumor cells. Thus, T-cell-redirecting bispecific antibodies (bsAbs), which combine the cell-specificity of MoAbs with the potency of T cells may be more effective and less toxic than either form of therapy alone. In animal experiments, the efficiency of such bsAbs has been well documented using anti-idiotypic specificities to target B-cell lymphomas.^{3,4} In human therapy, the most favored target antigen on B-cell malignancies actually is CD19.⁵⁻⁷ First phase I studies with intravenously administered CD3XCD19 bsAb showed only minor toxicity (grade I on the World Health Organization scale) and some evidence for efficacy was noted.^{6,7} However, because CD19 is also expressed on normal B cells and follicular dendritic cells, this approach is relatively unspecific. The absence of tumor-specific target antigens is a common problem for the majority of tumors.

To circumvent this problem, especially in the case of relapsing leukemia, we propose a new approach exploiting the chimerism between donor and recipient after mismatched allogeneic BMT. Thus, allogeneic BMT raises the possibility of redirecting donor T cells against recipient-type antigens on tumor cells by MoAbs or bsAbs. bsAbs have so far not been used in the context of BMT. A single-step purification

method previously reported provided a sufficient amount of quadroma-derived, intact bsAb for in vivo treatment.⁸

To test our approach in mice, we used two tumor models: (1) a thymoma tumor model using Thy-1.2 as an operationally tumor-specific antigen (in this case, the donor is Thy 1.1) and (2) a B-cell lymphoma tumor model using recipient-type major histocompatibility complex (MHC) class II as an operationally tumor-specific antigen. In the second model, we wanted to test the MHC class II antigen, which is stably expressed on a broad range of leukemias, as an alternative target in recurrent leukemia after MHC class II-incompatible BMT. Furthermore, we measured the cytokine release (interleukin-6 [IL-6] and tumor necrosis factor α [TNF α]) after different bsAb injection schedules to find a suitable application scheme.

MATERIALS AND METHODS

Mice

Ten- to 12-week-old female BALB/c (H-2^d, I-A^d, Thy-1.2) and 12- to 14-week-old female C57BL/6-Thy-1.1 (H-2^b, I-A^b, Thy-1.1) mice originally obtained from the Jackson Laboratory (Bar Harbor,

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Supported by Else-Kröner-Fresenius-Stiftung (Grant No. 77829). Address reprint requests to Horst Lindhofer, PhD, GSF-Institut für Immunologie, Marchioninistr. 25, 81377 München, Germany.

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0006-4971/96/8812-0014\$3.00/0

ME) were raised and maintained in our breeding facilities at the GSF-Forschungszentrum für Umwelt und Gesundheit. Mice were housed in groups of 4 to 6 animals in microisolator cages under normal conditions and received autoclaved food and acidified water ad libitum. CB-17 SCID/SCID (H-2^d, I-A^d, Thy-1.2; originally obtained from H. Wagner, University of Ulm, Ulm, Germany, with the kind permission of M.S. Bosma, Fox Chase Cancer Center, Philadelphia, PA) were bred and kept under specific pathogen-free (SPF) conditions.

Cells and Media

The following parental hybridomas were used for quadroma production and have been described previously: MmT1 (mouse IgG2a, antimouse-Thy1.2),⁹ 17A2 (rat IgG2b, antimouse-CD3),¹⁰ HB3 (mouse IgG2a, antimouse I-A^d; American Type Culture Collection [ATCC], Rockville, MD). BCL1 lymphoma (in vivo and in vitro) lines expressing I-A^d (MHC class II)¹¹ were kindly provided by Dr K. Thielemans (Brussels, Belgium). The thymoma ST-1 was isolated from a severe combined immunodeficient (SCID) mouse, passaged three times in BALB/c mice, and characterized as Thy 1.2⁺ and CD3⁻. The murine T-cell line LBRM-33 4A2 (ATCC) and BCL1 in vitro line were used for cytotoxicity assays. Cells were cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS) supplemented with antibiotics and L-glutamin.

Generation of Quadroma-Derived Antitarget × Anti-CD3 bsAbs

bsAbs G2 (CD3 × Thy-1.2) and BiC (CD3 × I-A^d) were produced as described earlier.⁸ The fusion of rat with mouse hybridomas allows the high yield production of bsAbs. Briefly, a hypoxanthin-aminopterin-thymidin (HAT)-sensitive hybridoma producing the 17A2 (anti-CD3, ratIgG2b) antibody was fused with the iodoacetamide-treated HAT-resistant MmT1 (anti-Thy-1.2, mIgG2a) or HB3 (anti-I-A^d, mIgG2a) hybridoma cell lines. After selection with HAT-containing medium, suitable clones, designated G2 and BiC, were established. As a result of preferential H/L pairing in rat/mouse quadromas, about 40% to 50% of the produced Ig was correctly paired bsAb. For purification, the culture supernatant was subsequently loaded onto a protein A column (Bio-Rad, Hercules, CA) and bsAb was sequentially eluted at a near physiologic pH of 5.7, whereas parental mouse antibodies were stably retained in the column. The column was regenerated by eluting the parental mouse antibodies at pH 3.5.

Cytotoxicity Assay

The cytotoxicity of cytotoxic T lymphocytes (CTLs) redirected by bsAbs was determined in a 6-hour ⁵¹Cr-release assay. Effector cells were generated by incubating mouse spleen cells with a CD3 MoAb (17A2) and IL-2 (200 U/mL) for 3 days. The activated effector cells were then cultured with ⁵¹Cr-labeled target cells (5 × 10³ cells) at various effector to target (E/T) ratios with 200 ng/well (1 μg/mL) of the appropriate bsAb or the parental MoAb, respectively, for 6 hours. In another setting, we used an E/T of 20/1 with the appropriate bsAb or the parental MoAb, respectively, in a serial dilution starting at 500 ng. BCL1 and LBRM were used as target cells. The percentage of specific lysis was calculated as follows: (Experimental Release – Spontaneous Release)/(Maximum Release – Spontaneous Release) × 100. Spontaneous release did not exceed 10% of the maximum release.

BMT and Tumor Model

Groups of 18 to 30 BALB/c mice were γ-irradiated with an effective dose of 6, 7, or 8 Gy from a ¹³⁷Cs source (HWM-D-2000;

Gammacell, Ottawa, Quebec, Canada); a skin-to-source distance of 35 cm; and a dose rate of 0.9 Gy/min. Four hours later, a combination of in vivo T-cell-depleting MoAbs (RmCD4-2 and RmCD8-2; both rat IgG2b) was injected intraperitoneally in a volume of 500 μL phosphate-buffered saline (PBS) to prevent graft-versus-host disease (GVHD) after MHC class I- and II-incompatible BMT, as previously described.¹² The animals received 200 μg of each MoAb. On day 0, C57BL/6–Thy-1.1 donor animals were aphyxiated using CO₂. BM cells were prepared by flushing the femurs and tibias with minimum essential medium (MEM)-Earle (Seromed, Berlin, Germany). The cell material was carefully resuspended and twice washed with medium, nucleated cells (NC) were counted after erythrocytes were lysed, and the cell suspension was adjusted with Dulbecco's PBS (Seromed) to 3 × 10⁷ NC/mL. Three hundred thirty microliters of this cell suspension containing 10 × 10⁶ C57BL/6 BM cells (BMCs) were injected intravenously via the lateral tail vein into the recipients 24 hours after irradiation. Syngeneic tumor cells ST-1 and BCL1 were inoculated in chimeras between days 40 and 50 after transplantation. Statistical evaluation of survival curves was performed using the logrank method.¹³

Fluorescence-Activated Cell Sorting (FACS) Analysis for Assessment of Chimerism and T-Cell Reconstitution

Chimerism and T-cell repopulation of the peripheral blood was analyzed in all animals at different time points after BMT by two-color flow cytometry. Samples of 50 μL of blood were taken with heparinized glass capillaries from the mice tails. The blood was flushed from the capillaries with 100 μL of medium, washed once with FACS buffer (DPBS [Seromed] containing 2 vol% heat-inactivated FCS [GIBCO-BRL, Life Technologies Inc, Grand Island, NY] and 0.1% sodium acid [Sigma, St Louis, MO]) and incubated for 20 minutes with 25 μL culture supernatant of the MoAb 2.4.G2 (rat-antimouse Fcγ receptor II and III; ATCC) to prevent unspecific binding of the following MoAbs. Afterwards, combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, or biotin-conjugated MoAbs were added in optimum concentrations and incubated for 30 minutes at 4°C. Unbound antibody was removed in a washing step and streptavidin-PE was added to probes containing a biotin-labeled MoAb. Finally, erythrocytes were lysed using lysis buffer and the remaining leucocytes were resuspended in FACS buffer. A combination of antibodies for MHC class I antigens of donor-type (H-2^d, FITC-conjugated, clone KH95; Pharmingen, San Diego, CA) or host-type (H-2^d, biotin-labeled, clone 34-2-12S; ATCC) was used for the assessment of chimerism. T-cell reconstitution after in vivo T-cell-depleted BMT was measured using a combination of MoAbs to CD3 (FITC-conjugated, clone 17A2) and CD4 (PE-conjugated, clone GK1.5; Becton Dickinson, Mountain View, CA) or CD3 and CD8 (PE-conjugated, clone YTS169.4; Caltag).

Cytokine Assay

In an effort to minimize possible toxic side effects of antibody-related T-cell activation, we injected 200 ng on day 3 (preinjection) and 10 μg of the parental antibodies or bsAb on day 4 after tumor inoculation. Twenty-five microliters of tail vein blood was taken 1 and 3 hours after antibody injection on day 4 and diluted with 100 μL MEM-Earle medium. After removing cells by centrifugation, sera were stored at –20°C.

For the quantitation of IL-6, a colorimetric assay¹⁴ was employed using the IL-6-specific mouse hybridoma cell line 7TD1¹⁵ as an indicator system. In brief, 1 × 10⁴ 7TD1 cells/mL were stimulated with serially twofold diluted test samples in flat-bottomed, 96-well microplate cultures (100 μL/well). After 4 days, MTT-formazan produced during the last 4 hours was measured as described pre-

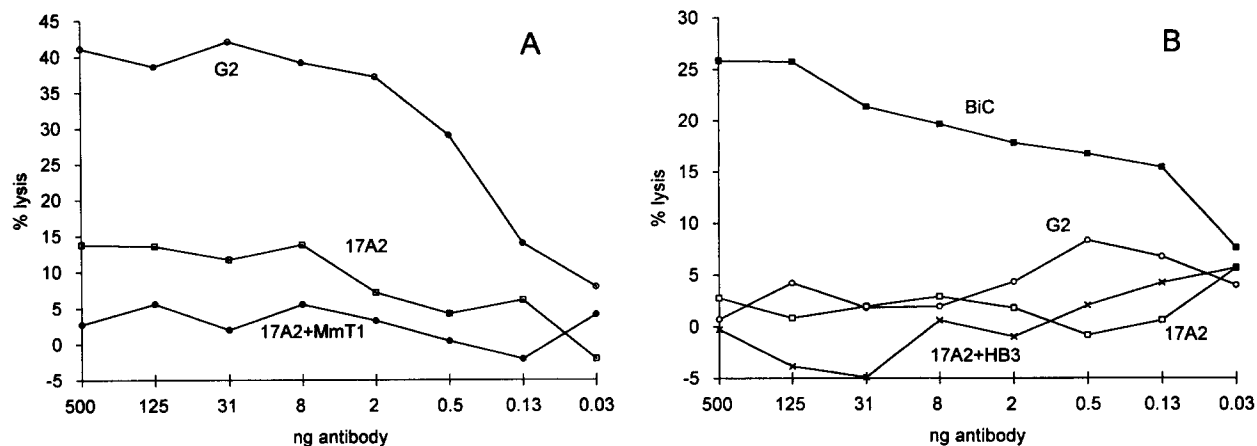


Fig 1. Redirected lysis of the LBRM (A) and BCL1 (B) tumor cells by preactivated spleen cells. LBRM tumor cell lysis in the presence of bsAb G2 (○), parental antibodies MmT1 (anti-Thy-1.2) and 17A2 (●), or parental antibody 17A2 (□). BCL1 tumor cell lysis in the presence of bsAb BiC (■), parental antibodies HB3 (anti-I-A^d) and 17A2 (×), parental antibody 17A2 (□), or bsAb G2 (○) (at an E:T ratio = 20:1).

viously.¹⁶ Recombinant human IL-6 (Promega Corp, Madison, WI) was used as a positive control. The specificity of the IL-6 assay was verified using a commercially available anti-IL-6 receptor antibody (Genzyme, Cambridge, MA). IL-6 concentrations were calculated from the linear parts of the dose-response curves defining 1 U/mL of IL-6 as the amount causing a half-maximum proliferative stimulation of 7TD1 cells in this assay. Serum samples were tested for biologically active TNF α ¹⁷ by assessing the cytotoxicity on L929 target cells in the MTT assay.

RESULTS

Specificity of the Cytolysis Induced by the BsAbs G2 and BiC

We produced two bsAbs with specificities anti-CD3 $\times$ anti-Thy-1.2 for the thymoma model and anti-CD3 $\times$ anti-I-A^d (class II haplotype of BALB mice) for the B-cell lymphoma tumor model. Before starting with in vivo experiments, we tested the cytolytic potential of the bsAbs in a ⁵¹Cr-release assay. Freshly isolated spleen cells were cultured for 3 days with IL-2 (200 U/mL) and coated anti-CD3 MoAb. The activated splenocytes were subsequently tested for their ability to lyse Thy-1.2⁺ LBRM cells or I-A^d BCL1 cells in the presence or absence of the bsAbs. As controls, we used the parental antibodies of the tumor target arms: anti-I-A^d and anti-Thy-1.2 in combination with the anti-CD3 antibody 17A2 and, as additional control, the bsAb G2 on the Thy-1.2⁺ BCL1 cell line. Figure 1 shows that control antibodies have only marginal cytolytic potential in this in vitro assay, whereas both bsAbs showed strong cytolytic activity.

Evaluation of Chimerism in BALB/c Mice Transplanted With C57BL/6 Thy-1.1 BM

About 10% to 20% of the mice died during the first 8 to 10 days after BMT and a total body irradiation (TBI) dose of 8 Gy, probably due to radiation injury or nonengraftment. Surviving mice became stable chimeras and survived for

more than 200 days after BMT without signs of acute or chronic GVHD. A reduction of the TBI dose to 7 or 6 Gy reduced mortality to 0%, but, when chimerism was measured, we found a mixed chimerism. Because the aim of this study was to determine the effectiveness of redirecting donor T cells to host-type antigens on leukemic cells, we preferred chimeras with nearly 100% donor-type hematopoiesis and decided to work with the higher dose of 8 Gy TBI despite a loss of animals.

T-Cell Reconstitution

As shown in Fig 2, T-cell reconstitution in C57BL/6 Thy-1.1 into BALB/c chimeras is rather slow after in vivo T-cell-depletion with a combination of MoAbs against CD4 and CD8. Two to three weeks after BMT, the percentage of CD3⁺ blood T lymphocytes is less than 3%. Between 3 and 6 weeks after BMT, donor T lymphocytes are slowly increasing. On day 40 after BMT, 13.1% ($\pm$ 2%) of the peripheral blood lymphocytes (PBLs) were CD3^{bright}/CD4^{bright}.

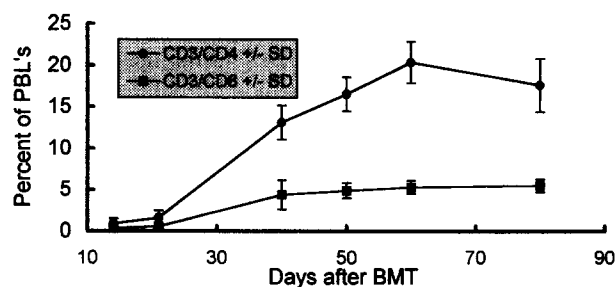


Fig 2. T-cell reconstitution in C57BL/6 Thy-1.1 into BALB/c chimeras after 8 Gy TBI and protection with T-cell-depleting antibodies. Percentages of CD3/CD4 double-positive (●) and CD3/CD8 double-positive T cells (■) of the PBLs as determined by FACS analysis at different time points after BMT. n = 6; error bars indicate standard deviations. Data are from one of three consecutive experiments with similar results.

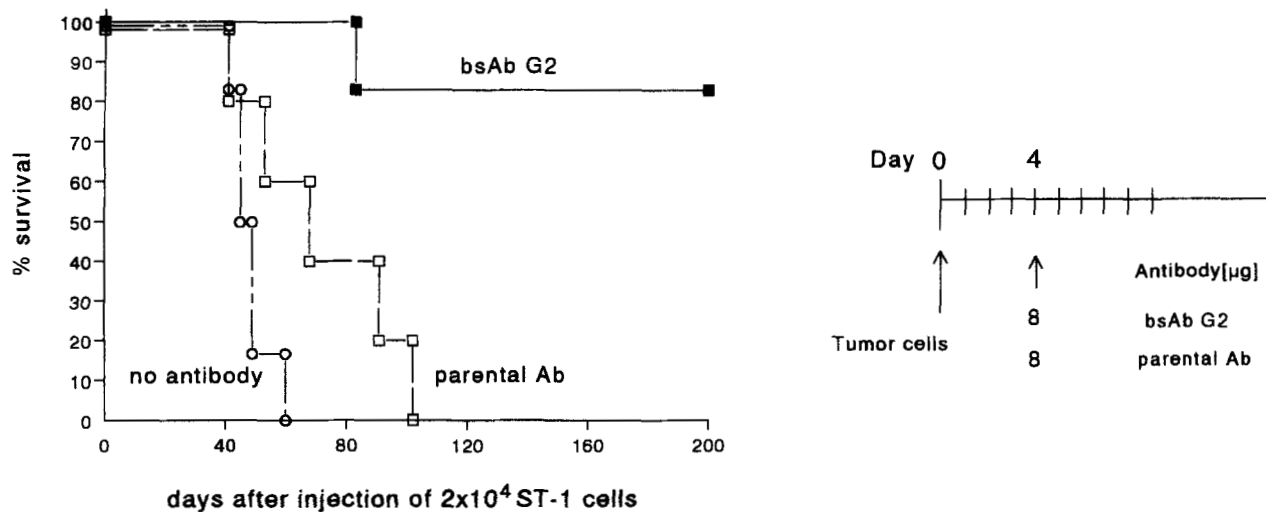


Fig 3. Survival of mice after inoculation with 2×10^4 ST-1 cells and treatment with bsAb G2 (■), parental antibody MmT1 (anti-Thy-1.2; □), or no antibody (○). Statistics: survival parental antibody versus no antibody ($P = .15$), bsAb versus no antibody ($P < .003$), and bsAb versus parental antibody ($P < .01$).

positive and 4.3% ($\pm 1.8\%$) were CD3^{bright}/CD8^{bright}-positive T cells. The percentage of CD4⁺ cells increased up to 20% of the PBLs within 60 days after BMT. The percentage of CD8⁺ cells was slowly increasing from 4.3% ($\pm 1.8\%$) at day 40 to 5.5% ($\pm 0.8\%$) on day 80 after BMT. Overall, the percentage of T cells among the PBLs remains low in C57BL/6 into BALB chimeras and never exceeds 30%. Because absolute numbers of leukocytes are not higher in C57BL/6 into BALB chimeras than in untransplanted BALB mice (data not shown), the chimeras remained lymphopenic after BMT.

Therapeutic Effect of bsAbs G2 and BiC on Injected Tumor Cells

bsAb G2. Because bsAbs showed cytolytic potential in the ⁵¹Cr-release assay and transplanted mice had a stable chimerism of almost 100%, we started with in vivo experiments. First, we tested the thymoma tumor model using the bsAb G2. Six weeks after BMT, 2×10^4 recipient-type, T-cell-derived ST-1 tumor cells were injected. Four days later, mice received 8 μ g of bsAb G2 (anti-CD3 $\times$ anti-Thy-1.2). This combination guaranteed an exclusive binding of the target arm of the bsAb to tumor cells in the chimeras, because new hematopoiesis generated only donor-type Thy-1.1⁺ cells. Compared with the parental anti-Thy-1.2 antibody, the bsAb reduced tumor mortality markedly. Only 1 of 6 mice died in the bsAb group, clearly documenting the benefit of the redirection principle (Fig 3). This was especially interesting, because the parental anti-Thy-1.2 antibody by itself is a very potent T-cell-depleting antibody.¹⁸

bsAb BiC. As next step, this approach was extended to a clinically more relevant target antigen. We addressed the question of whether B-cell lymphomas can be targeted by an operationally tumor-specific antigen such as MHC II (I-A^d) after allogeneic BMT. In many cases, B-cell lymphomas express MHC II strongly and stably. Therefore, MHC II polymorphism

can easily be exploited to target B-cell lymphomas by recipient class II-specific MoAb or bsAb, even after an MHC class I-matched but class II-mismatched BMT.

In our experiments, we transplanted BALB/c mice with C57BL/6 BM so that new hematopoiesis generates only I-A^b-positive antigen-presenting cells (APCs) or B lymphocytes. Thus, the reconstituted immune system would not be affected by this therapy. In a preliminary experiment, we injected 10^3 BCL1 B-cell lymphoma cells and treated mice with a relatively high bsAb dose (42 μ g) of the anti-I-A^b anti-CD3 bsAb BiC to test the antitumor efficacy and possible toxic side effects. The parental antibody control was equimolar to the bsAb. In this experiment, only 50% of the parental antibody control group survived, whereas all mice of the bsAb group survived (Fig 4A). Encouraged by this first experiment, we increased the tumor load (20-fold) to 2×10^4 tumor cells and decreased the bsAb dose to 20 μ g (see exact application in Fig 4). Furthermore, we waited 4 days after tumor injection before injecting antibodies.

As a result of these rather harsh conditions, only 2 of 6 mice survived from the bsAb group. In comparison with the two parental antibodies (17A2 and HB3) and the transplanted control group, the benefit of the bsAb is still clearly visible and significant ($P < .009$ and $P < .003$, respectively; Fig 4B). Remarkably, the addition of the parental anti-CD3 antibody to the parental anti-class II antibody in the BCL1 tumor model was still not able to prolong the survival significantly ($P = .08$) versus the tumor control group, which is comparable to the ST-1 experiments in which we injected the parental anti-Thy-1.2 antibody alone. A preinjection of 200 ng bsAb at day 3 after tumor inoculation in 3 of 6 mice (to investigate a possible influence upon cytokine release) appeared to have no effect on the survival, because 1 of the surviving animals was preinjected and the other was not. The evaluation of body weights showed that, in the preliminary high-dose experiment (42 μ g bsAb),

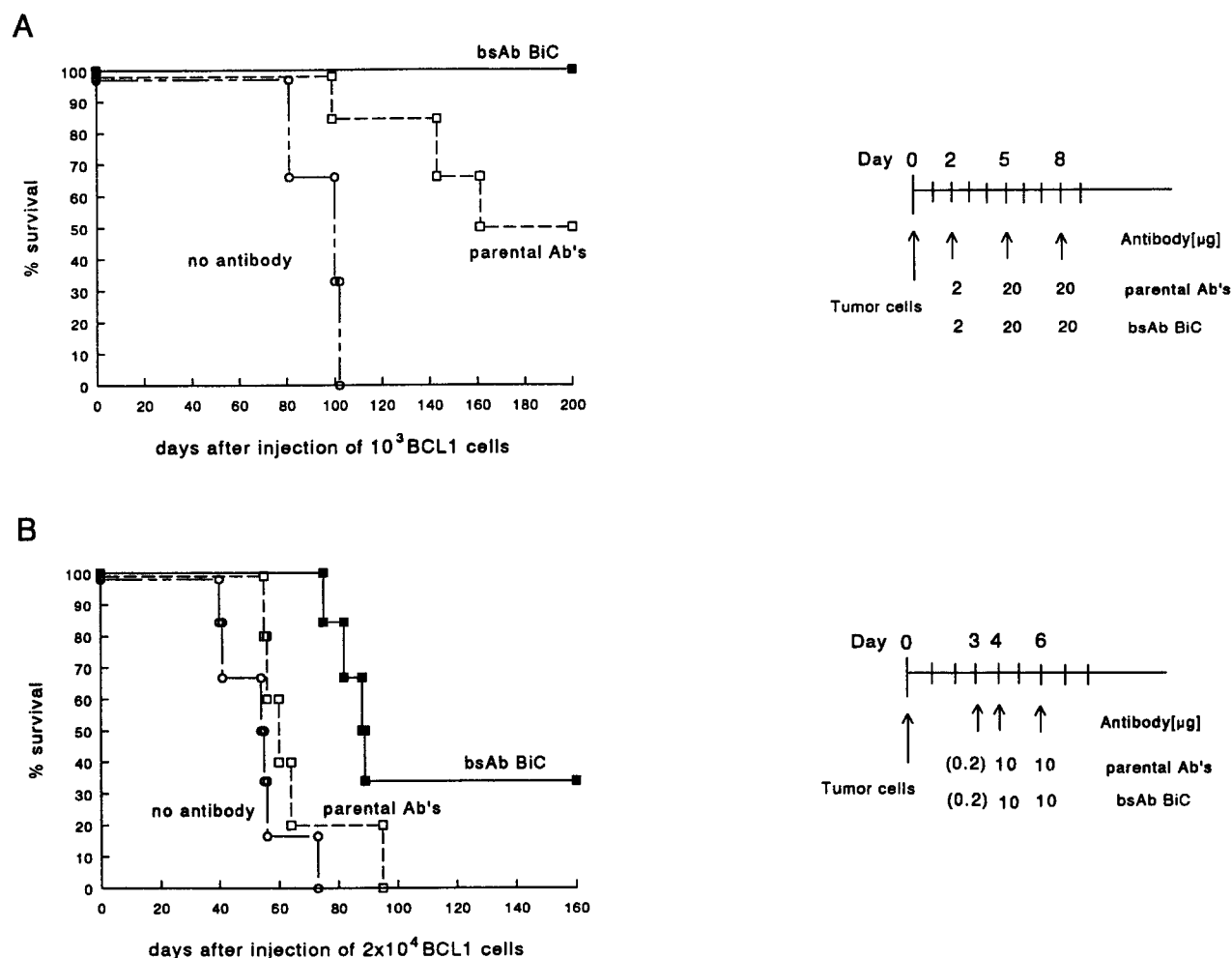


Fig 4. Survival of mice after inoculation with (A) 10^3 or (B) 2×10^4 BCL1 cells and treatment with bsAb BiC (■), parental antibodies HB3 and 17A2 (anti-I-A^d, anti-CD3; □), or no antibody (○). Statistics: (A) parental antibodies versus no antibody ($P = .08$), bsAb versus no antibody ($P < .005$), and bsAb versus parental antibodies approached significance ($P = .056$). (B) Three of six mice were preinjected at day 3 with 200 ng of BiC or parental antibodies (100 ng each). Statistics: parental antibody versus no antibody approached significance ($P = .078$), bsAb versus no antibody ($P < .003$), and bsAb versus parental antibody ($P < .009$).

mice lost 10% of weight, compared with about 5% weight loss in the 2×10^4 bsAb experiment without preinjection and only about 1% weight loss with preinjection (data not shown). Obviously, 42 μ g of intact Fc-bearing bsAb with anti-CD3 binding arm (which corresponds to a dose of 2 mg/kg) approximates the tolerated dose in mice.

Cytokine Release After Different MoAb or BsAb Application Schemes

Toxic side effects such as weight loss and slight diarrhea were observed in the first B-cell lymphoma experiment (42 μ g of bsAb, Fig 4A). In an effort to minimize the toxic side effects, we tested the possibility of whether small amounts of preinjected anti-CD3 containing antibody could decrease the first dose cytokine response.¹⁹ To find a possible correlation between antibody doses, application of antibody, and the observed weight loss, we determined the cytokine release at differ-

ent time points. In the second B-cell lymphoma experiment, tail vein blood was taken 1 and 3 hours after the 10 μ g antibody injection on day 4 to determine the IL-6 and TNF α release. Furthermore, 3 of 6 mice in both groups were preinjected with 200 ng of a combination of parental antibodies or bsAb on day 3 after tumor inoculation (Fig 4B). Interestingly, no TNF α was detected in the peripheral blood after the injection of 10 μ g (0.5 mg/kg) parental antibodies or bsAb. In contrast, IL-6 release could be assessed, and differences in release indicate a correlation between preinjection of low doses of either parental anti-CD3 antibody or bsAb and subsequent cytokine release on the following day after injection of 10 μ g parental antibodies or bsAb, respectively. The exact cytokine release patterns for both groups are shown in Fig 5.

DISCUSSION

Actually, two therapies exist to efficiently treat leukemia relapse after BMT: a second BMT or donor lymphocyte

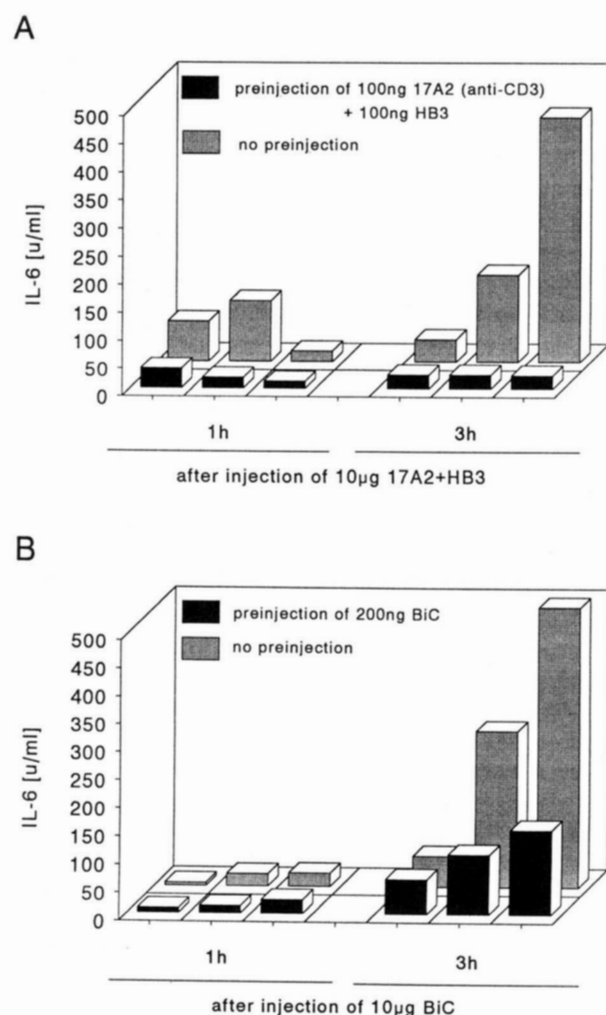


Fig 5. Effect of preinjection of anti-CD3 antibody 17A2 (A) or anti-CD3 containing bsAb BiC (B) on IL-6 release in mice. Three of six mice of the parental antibody or bsAb group were preinjected with the indicated doses (100 ng 17A2 or 200 ng BiC) 3 days after tumor inoculation. On day 4, all mice received 5 μ g 17A2 + 5 μ g HB3 (A) or 10 μ g BiC (B) of the respective group (see Fig 4 application scheme). Tail vein blood was taken 1 and 3 hours after antibody injection. Columns represent individual mice.

infusion (DLI). Both have certain disadvantages. A second BMT is a more serious life-threatening intervention, whereas DLI, being remarkably efficient in the therapy of chronic myelogenous leukemia (CML), carries the risk of uncontrollable graft-versus-host reactions.¹ Having overcome difficulties in the generation of quadroma-derived bsAb amounts sufficient for in vivo studies,⁸ we here introduce a new immunotherapeutic approach using MoAb or more favorable bsAbs that exploit the class II polymorphism or serologically defined differences between donor and recipient. It does not depend on as yet little defined tumor or minor histocompatibility antigens and is not limited by MHC restriction.

We show the efficiency of this approach in two different

mouse leukemia relapse models after allogeneic BMT using the Thy-1 dimorphism in mice and class II polymorphism in analogy to the human situation. An important prerequisite before starting with therapy experiments is an established chimerism. Evaluation of the chimerism after allogeneic BMT showed that all mice transplanted were about 99% chimeric. T-cell reconstitution lasted more than 60 days in this fully incompatible C57BL/6 in BALB/c transplantation model. Forty-five days after BMT, when therapy was started, only about 20% of lymphoid cells were CD3⁺ (15% CD4, 5% CD8). Nevertheless, in the case of the ST-1 T cell lymphoma, bsAb G2 efficiently redirected the T cells for tumor destruction irrespective of the low T-cell number. In the bsAb group, 84% of the mice survived, whereas the T-cell-depleting¹⁸ parental (anti-Thy-1.2) antibody MmT1 could only prolong survival.

In this model, we injected only the anti-Thy-1.2 antibody MmT1 to determine the antitumor efficacy of this potent T-cell-depleting antibody alone. Certainly, in our experimental setting (allogeneic BMT reconstituting a Thy-1.1 hematopoiesis in Thy-1.2 mice), the anti-Thy-1.2 antibody has a much more potent effect on the Thy-1.2 tumor than an equivalent amount (4 μ g) of 17A2 antibody. This small amount of anti-CD3 antibody would not be expected to cause significant antitumor efficacy.^{20,21} Moreover, we show in our second tumor model (Fig 4) that the mixture of parental anti-CD3 and anti-class II antibodies did not significantly prolong the survival of tumor-treated mice compared with the untreated control. Thus, our data indicate that the unspecific T-cell activation by small amounts of anti-CD3 antibody could not significantly inhibit tumor growth in vivo.

The relatively low aggressiveness of the ST-1 tumor line might be one reason for this successful therapy experiment. In contrast to ST-1, the BCL1 lymphoma is more aggressive due to multiple passages over the last 18 years.¹¹ Therefore, the low T-cell number in the chimeras could be more critical for the therapy outcome of the B-cell lymphoma model and might be one reason that only 34% of mice survived although a more than twofold bsAb dose was delivered. Another important point for the relatively low survival in the BCL1 tumor model could be the class II expression, which is 10-fold less compared with the Thy-1.2 expression on ST-1 cells, as determined by FACS analysis (data not shown). We have started experiments to target also the I-E^d (corresponds to HLA-DR) besides the I-A^d (HLA-DQ) antigen to increase the antibody density on the BCL1 tumor cells and improve survival.

Another point of interest was the evaluation of possible negative side effects of this antibody-based, immunotherapeutic approach. Even in the high-dose bsAb group (42 μ g/mouse at 2 mg/kg), no animal died due to toxic side effects of either the anti-CD3 or anti-class II antibody binding arm. However, a weight loss of about 10% indicates some toxicity. Because cytokines released by activated T cells or accessory FcR⁺ cells might be responsible for toxic side effects, we measured TNF α and IL-6 after antibody injection. To our surprise, we could not detect TNF α after the injection of 0.5 mg/kg intact MoAb or bsAb. However, IL-6 release

was easily detectable and our results suggest that, by using low-dose preinjections (100 ng and 200 ng) of anti-CD3 parental antibody or anti-CD3 containing bsAb, the cytokine release can be markedly decreased after a subsequent high-dose application (5 μ g and 10 μ g) 24 hours later. Thus, for bsAb treatment, the cytokine release has been diminished from at average 281 U in absence to 106 U in presence of preinjected bsAb (200 ng). For parental anti-CD3 antibodies, this effect was even more dramatic. IL-6 release decreased 10-fold from at average 211 U without preinjection to 25 U with preinjection of anti-CD3 antibody (Figs 4B and 5A). Interestingly, the decrease of cytokine release after preinjection had no consequence for the antitumor efficiency of the bsAb therapy. Therefore, the dose escalation of anti-CD3-containing bsAb (Fig 5B) seems to be a practicable application to avoid negative side effects such as the first dose cytokine response¹⁹ in bsAb-mediated immunotherapy. Initial phase I studies using a similar application scheme with dose escalating bsAb doses for immunotherapy of B-cell lymphomas resulted only in grade I toxicity.^{6,7}

As expected, donor B cells were not affected by recipient class II-directed immunotherapy. This may prove an advantage over the targeting of B-cell lymphomas by, eg, nonpolymorphic CD19, which cannot spare B cells and follicular dendritic cells.⁷ But how specific is MHC class II as a tumor target antigen after allogeneic BMT? In the early phase after BMT, a variety of cytokines is released, such as interferon- γ , which is also responsible for class II expression on endothelial and epithelial cells. At this time, anti-MHC class II tumor therapy with antibodies would be rather unspecific and could support a graft-versus-host reaction. But, in most cases, the leukemic relapse comes later, when cytokine levels have been normalized²² and MHC class II expression is again downregulated in susceptible tissues. In this situation, the benefit of a nonmodulating, stable, and densely expressed target antigen such as class II on the tumor cells should become evident.

HLA-A, -B, and -DR matching is a necessary and useful prerequisite to prevent GVHD after allogeneic BMT.²³ The MHC class II loci DQ and DP are not routinely typed because mismatches in these loci are mostly tolerated and because of the lack of a suitable, fully matched donor in most cases.

In contrast to MHC class I expression, which is often downregulated on malignant clones, MHC class II expression is even increased in the majority of leukemias such as B-cell acute lymphoblastic leukemia, acute myeloid leukemia (except subtype M3, French-American-British classification), CML, and lymphomas.^{24,25} This is also true for some solid tumors, such as larynx or cervix carcinoma, and, in some cases, malignant melanoma or colon and breast carcinoma.²⁶ Therefore, the class II antigens DQ and DP could be an attractive target antigen for immunotherapy with MoAbs or bsAbs in allogeneic BMT. The present approach may also gain importance in leukemias treated with cord blood transfusions in which HLA mismatches are better tolerated or in haploidentical family BMT, which seems to be practicable using high numbers of stem cells.²⁷ Another

indication could be a more specific immunosuppression of the host-versus-graft reaction in organ transplantation when donor BM is cotransplanted. In this case, recipient specific anti-class II antibodies would not affect the donor hematopoiesis, but selectively attack recipient-type activated T cells.

In conclusion, we have shown that polymorphic antigens on tumor cells can be operationally tumor-specific and targeted by MoAbs or bsAbs with the consequence of improved survival of BM chimeras.

ACKNOWLEDGMENT

The authors thank S. Breuer and H. Broszeit for excellent technical assistance, Dr E. Holler for helpful discussion, and Dr N. Sidell for critically reading the manuscript.

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