Prevention of Epstein-Barr Virus-induced Human B-Cell Lymphoma in Severe Combined Immunodeficient Mice Treated with CD3xCD19 Bispecific Antibodies, CD28 Monospecific Antibodies, and Autologous T Cells

Heribert Bohlen, Oliver Manzke, Sandra Titzer, Johann Lorenzen, Dieter Kube, Andreas Engert, Hinrich Abken, Jürgen Wolf, Volker Diehl, and Hans Tesch


ABSTRACT

Bispecific CD3 × antitumor antibodies in combination with coactivating CD28 antibodies can induce resting T cells to proliferate and to lyse syngeneic tumor cells (M. Azuma et al., J. Immunol., 150: 2091—2101, 1993; M. Azuma et al., J. Exp. Med., 177: 845—858, 1993). This combination antibodies may therefore be useful for active immunotherapy of malignant tumors. In this study, we present a preclinical model to evaluate CD3xCD19 bispecific antibodies. We investigated whether bispecific antibodies prevent the development of malignant EBV-induced lymphomas in severe combined immunodeficient (SCID) mice which lack functional B and T lymphocytes (G. C. Bosma et al., Immunogenetics, 29: 54—57, 1989). SCID mice were engrafted with peripheral blood lymphocytes and EBV and treated after 3 days with CD3xCD19 bispecific antibodies and CD28 antibodies. Our data demonstrate that the growth of B cells lymphomas can be prevented in SCID mice by treatment with CD3xCD19 bispecific antibodies and that B-lymphoma-specific T cells can be recruited. In contrast to in vitro experiments, there was no clear effect of CD28 administration which is due to high expression of B7—1 on the transplanted B cells. Lymphoma-bearing mice had elevated titers of interleukin10 in the serum, in contrast to tumor-free animals. As shown by PCR analysis, there was no evidence of dormant B-lymphoma cells in specimens from surviving mice. In the spleen of surviving mice, rearranged human T-cell receptor γ gene segments were detectable. Furthermore, mice that were initially treated with CD3xCD19 and CD28 antibodies did not develop lymphomas upon rechallenge with EBV-infected mononuclear cells of the same donor, whereas control animals did. Our results obtained from this autologous human B-lymphoma model have implications for the design and evaluation of new immunotherapeutic modalities for the treatment of human B-cell lymphoma with bispecific antibodies.

INTRODUCTION

Bispecific anti-T-cell × antitumor monoclonal antibodies can recruit T cells to kill autologous tumor cells (1, 2). The protection against tumor growth by bispecific antibodies has been described in the syngeneic 38C13 and BCL-l B-cell lymphoma model (3, 4, 5, 6). In these experiments, established lymphoma could be cured by treatment with CD3 × anti-idiotypic antibodies. In addition to the recruitment of activated T cells by CD3 × anti-idiotypic antibodies, effector cells, like natural killer cells, can also be redirected to a given tumor. This was demonstrated with bispecific antibodies consisting of a CD16-binding moiety and a tumor-specific binding site (7, 8, 9). A common requirement of these therapy modalities, however, is the dependence on preactivated effector cells. Jung et al. (10) showed, in a melanoma model, that resting T cells could be activated by CD28 costimulation. In addition, CD3xCD19 bispecific and CD28 monospecific antibodies activated resting T cells in B-cell chronic lymphocytic leukemia patients as shown in previous studies (11).

SCID mice transplanted with human PBMCs exhibit functional components of the human immune system inducing immunoglobulin production and circulating CD3-positive T cells (12) which persist in liver and spleen for several months. SCID mice transplanted with PBMCs from EBV-positive donors, however, develop aggressive human B-cell malignancies at a high frequency (13, 14).

In this study, we determined whether autologous human T cells can be activated with CD3xCD19 bispecific and CD28 monospecific antibodies and prevent the development of EBV-induced B-cell lymphomas in PBMC-transplanted SCID mice.

MATERIALS AND METHODS

Cells and Bispecific Antibodies. The hybridoma OKT3 (CD3) (American Type Culture Collection, Rockville, MD) was kindly provided by Dr. K. Thielemans (Free University of Brussels, Brussels, Belgium), and the hybridoma cell line 15E8 producing antibodies specific for the CD28 homodimer molecule was provided by Dr. R. van Lier (Ref. 15; Netherland Red Cross, Amsterdam, the Netherlands). All hybridoma cell lines were grown in RPMI 1640 (Life Technologies Inc., Karlsruhe, Germany) supplemented with l-glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and 1% FCS (Life Technologies, Inc.).

The generation and isolation of bispecific antibodies from tetradoma supernatant has been described elsewhere (11, 16). Briefly, the tetradoma cell line 6A4xOKT3 (anti-CD19 × anti-CD3), HD239 × 15E8, and HD239meg × 15E8 and the hybridoma cell line 15E8 (anti-CD28) were grown in hollow-fiber bioreactors. The supernatants were concentrated by ultrafiltration and purified by single-step hydrophobic interaction chromatography on phenyl-superose columns. Antibody preparations were tested for bi-isotypic antibodies using a double-isotype ELISA. The preparations were checked by isoelectric focusing and SDS-PAGE. The bispecific binding of the antibody preparations was confirmed by its ability to stimulate T lymphocytes and to redirect cytotoxicity of cytotoxic T cells to CD19-positive B-cell lines as described previously. Before therapeutic usage, the antibody preparations were checked for endotoxin contamination using the Limulus assay. Specimens exceeding 2 EU/ml were treated with Triton X-114.

Isolation of PBMCs and Reconstitution of SCID Mice. Human PBMCs were obtained from healthy EBV-seropositive donors. For SCID mouse engraftment, PBMCs were resuspended to a concentration of 2.5 × 10⁸ cells/ml in supernatant of the EBV-secreting cell line B95/8. SCID mice (ages 5—7 weeks) were screened for the presence of mouse immunoglobulins using a standard ELISA and leaky animals (immunoglobulin >5 μg/ml) were excluded from the following studies. SCID mice (10 animals/group) were engrafted with human PBMCs by i.p. injection of 5 × 10⁷ cells in 200 μl of EBV-containing medium (B95/8 supernatant). Mice used in the rechallenging experiment were chosen to be age matched (100 days).

EBV-transformed B-cell lines were generated by culturing 5 × 10⁶ cells/ml of B95/8 supernatant supplemented with 5 μg/ml cyclosporin A in 25-cm² cell
Table 1  PCR primer for amplification of EBV-specific and TCR-(J, V) specific sequences

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<tr>
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<td>J segments</td>
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<tr>
<td>JGT12</td>
<td>5'-AAG TGT TGT TCC ACT GCC AAA-3'</td>
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<td>JGT3</td>
<td>5'-AGT TAC TAT GAG CTT ACT CC-3'</td>
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<td>JGT4</td>
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<td>5'-CTC CTG CAG ATG ACT CCT ACA ACT CCA AGG TTG-3'</td>
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<td>5'-CTG CTG CAG ATG ACT CCT ACA ACT CCA AGG TTG-3'</td>
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Light Chain Mismatches Do Not Affect the Redirecting Capac

toward the cross-linking effects of bispecific antibody.

Fig. 1. Evaluation of light chain mismatches toward the cross-linking effects of bispecific antibodies. Autologous primary T cells (1 X 10^6/well) were coincubated with tetramer-negative B- and T-cell subsets derived from erythroleukemia cells (1 X 10^6/well) to evaluate the effects of the light chain mismatches on the redirecting capacities of the bispecific antibodies.

Culture flasks at 37°C in 8% CO2, Transformed B cells were subsequently subcloned and analyzed by FACS for the expression of B- and T-cell differentiation markers CD19, CD20, CD3, CD2, and CD7 and for CD2 and CD7 and by PCR for the presence of EBV-related gene products. The cells were used for rechallenging experiments when the final preparations were free of contaminating T cells, as detected by FACS (<0.1% CD3-positive cells).

Preparation of T Cells. T cells were enriched by passage over nylon-wool (treatment schedule 1B). There was no B-cell contamination as shown by FACS analysis with B-cell-specific monoclonal antibodies (CD19, CD20, CD21, and CD23; data not shown).

DNA and RNA Preparations. DNA and plasmid DNA were isolated according to standard methods. For RNA dot blot analysis, 2 X 10^6 cells were lysed in a buffer containing 50 mM Tris (pH 7.2), 10 mM EDTA, 3 mM MgCl2, 1000 units/ml human placenta RNAse (Life Technologies, Inc., Karlsruhe, Germany), and 0.2% NP40 at 4°C. The lysate was centrifuged twice at 8000 x g for 2 min. The supernatant was added to the same volume of a solution containing 20X SSC and 15% formaldehyde and incubated at 60°C for 15 min. The lysate (equivalent to 10^6, 3 X 10^6, 10^7, and 3 X 10^7 cells) was dotted onto Hybond-N membrane using minifold apparatus (Schleicher & Schuell, Dassel, Germany). Isolated DNA restriction fragments were labeled with 32P by the Klenow DNA polymerase reaction randomly primed with oligonucleotides. For hybridizations, the following cloned DNA fragments was isolated from vector DNA sequences before radioactive labeling: 1.7-kb CD7 cDNA, 1.2-kb Pstl fragment of pGAP dehydrogenase.

PCR Analysis. DNA was isolated from snap-frozen tissue material and subsequently analyzed for EBV-specific sequences. The PCR was initiated after heating the samples to 95°C for 5 min in PCR buffer by the addition of Taq polymerase and primer (Table 1). Twenty-five cycles were performed (annealing at 55°C for 30 s, extension at 72°C for 30 s, and denaturation at 95°C for 30 s) using a MWG Thermocycler. The final reaction product was analyzed by agarose gel electrophoresis. These preparations consist of functional bispecific antibodies (50—60%) and nonfunctional light chain mismatches (2 X 20—25%).

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Flow Cytometric Analysis. Mononuclear cells from the peritoneal lavage (peritoneal cavity was washed with at least twice with 2 ml of prewarmed sterile PBS) were isolated by Ficol density centrifugation (400 x g for 20 min at room temperature). One hundred μl of cell suspension (1 X 10^6 cells/test) were incubated with FITC-labeled CD45 and phycoerythrin-labeled CD19 antibodies for 25 min on ice. After two washes, the stained cells were analyzed on a FACScan using Lysis II software. A minimum of 1 X 10^4 events was acquired for each sample.

Analysis of Human Cytokines. Human cytokines of transplanted SCID mice were analyzed in sera obtained by retroorbital sinus bleeding. Sequential ELISA assays for hIL-2 (Quantikine; R&D Systems), hIL-6 (Quantikine; R&D Systems), human IFN-γ (Cytoscreen; Biosource International, Wetlake Village, CA), and hIL-10 (Cytoscreen; Biosource) were performed (50 μl of mouse serum/test). Sensitivity of ELISA assays was <6 pg/ml, showing cross-reactivity to neither other human nor the corresponding mouse cytokines. The sensitivity of the hIL-10 ELISA (detecting natural and recombinant hIL-10) was 5 pg/ml, with no cross-reactivity to viral IL-10 (BCRF-1 product of EBV) and approximately 1% cross-reactivity to mouse IL-10.

RESULTS

Light Chain Mismatches Do Not Affect the Redirecting Capacity of Bispecific Antibodies. Bispecific antibodies used in the present study were isolated from tetradoma supernatants by hydrophobic interaction chromatography. These preparations consist of functional bispecific antibodies (50—60%) and nonfunctional light chain mismatched antibodies (2 X 20—25%).

To evaluate the effects of the light chain mismatches on the redirecting capacities of the bispecific antibodies, we analyzed two tetradoma subclones of the same fusion HD239 X 15E8 (CD2OxCD28), one having lost the light chain gene for the CD2O-binding moiety. T cells were coincubated with B7-negative tumoral B cells, CD3xCD19, and CD2OxCD28 or with a 5-fold excess of mismatched matched antibodies (2 X 20—25%).
PREVENTION OF EBV-INDUCED B-CELL LYMPHOMA IN SCID MICE

Fig. 2A. Treatment of PBMC-challenged SCID mice with bispecific CD3xCD19 and CD28 antibodies. Antibody solutions were given on day 3 after human PBMC engraftment (150 μl i.p., 150 μl i.v., 50 μg of each indicated antibody). The survival was recorded. ●, group treated with PBS; ○, group treated with bivalent parental (CD3 + CD19 + CD28) antibodies; ■, group treated with CD3xCD19 bispecific antibodies; and ▲, group treated with CD3xCD19 bispecific and CD28 monospecific bivalent antibodies. Each group consisted of 10 mice. Before the experiments, the antibodies were checked for endotoxin contamination using the Limulus assay. B, Challenge of T-cell engrafted mice with EBV-transformed lymphoplasmoid B cells. Evaluation of natural EBV-reactive T cells. Animals were engrafted with nylon-wool-purified human T lymphocytes (1.5 × 10^7 cells i.p.); ●, group was additionally engrafted with 2.5 × 10^7 irradiated autologous EBV-transformed B cells (i.p.). On day 26, the mice were challenged with 1 × 10^7 autologous EBV-transformed B cells (i.p.). ■, on day 28, the mice in this group were treated with monospecific bivalent CD3, CD19, and CD28 antibodies; ○, this group was treated with bispecific CD3xCD19 and monospecific bivalent CD28 antibodies; ●, this group did not receive any additional treatment. The survival was recorded. Each group consisted of 10 mice. PBL, peripheral blood lymphocyte; LCL, lymphoblastoid cell line.

Combinations on T-cell proliferation was demonstrated after 4 days by means of [3H]thymidine incorporation analysis. As shown in Fig. 1, addition of mismatched CD20misxCD28 antibodies to a culture with CD3xCD19 and functional CD20xCD28 resulted in only a minor decrease of the costimulating effects of the CD20xCD28 bispecific antibodies.

CD3xCD19 Antibodies Protect against Lymphoma Growth in SCID Mice. SCID mice transplanted with PBMCs from an EBV-seropositive donor were subsequently superinfected with EBV (B95–8 supernatant). After 3 days the animals were treated with (a) buffer; (b) parental CD3, CD19, and CD28 monospecific bivalent antibodies; (c) CD3xCD19 bispecific antibodies; or (d) CD3xCD19 bispecific antibodies in combination with CD28 antibodies, respectively. As shown in Fig. 2A, all mice treated with buffer or parental antibodies died with signs of disseminating tumors within 60 days. However, the majority of animals treated with CD3xCD19 antibodies (50%) or CD3xCD19 bispecific antibodies in combination with CD28 (70%) antibodies survived without signs of tumor. Thus, it appears

Fig. 3. Detection of human B cells in PBMC-SCID mice by flow cytometric analysis. Left panel, Analysis of peritoneal cells from a mouse treated with buffer (PBS). Right panel, Analysis of peritoneal cells from a mouse treated with CD3xCD19 bispecific and CD28 monospecific bivalent antibodies. The peritoneal cavity of each mouse was washed with 2 × 2 ml of prewarmed PBS.
that these bispecific antibodies mediate protection from lymphoma growth in the presence or absence of CD28 antibodies. Because B7-1 is highly expressed on EBV-positive B-lymphoma cells and the blocking of B7-1 and B7-2 molecules (18–20) in vitro abolished the stimulation of EBV-positive B cells by CD3xCD19 bispecific antibodies alone, it is likely that the second signal for T-cell activation is delivered by the B7 molecule on the surface of EBV-transformed B cells.

To determine whether EBV-specific T cells from normal donors can survive in SCID mice and protect against subsequent lymphoma growth in the absence of bispecific antibodies, SCID mice were given injections of nylon-wool-enriched human T cells and then challenged with irradiated EBV-transformed B cells. One experimental control group (group 4) was additionally immunized with lethally irradiated autologous EBV-transformed B cells to enhance the T-cell response. After 26 days, the mice were challenged with autologous EBV-immortalized B cells. One group was treated with bispecific antibodies and monovalent CD28 antibodies. As shown in Fig. 2B, none of the SCID mice given injections of T cells alone survived the challenge of EBV-induced B-lymphoma cells. Thus, this control experiment clearly demonstrates that lymphoma protection can only be achieved if T cells, EBV-transformed B cells, and bispecific antibodies are administered simultaneously (Fig. 2A).

**Immunological Analyses.** Survival correlated with the absence of CD45/CD19 double-positive human B cells as shown by flow cytometric analysis of the peritoneal lavage of transplanted SCID mice, which could be detected in lymphoma-bearing animals but not in lymphoma-free animals given injections of bispecific antibodies (Fig. 3). CD45/CD19-positive cells were not detected in the peripheral blood.

Because CD3xCD19 plus CD28 antibodies induce cytokine production by resting T cells in vitro, we measured the levels of cytokines (IL-2, IFN-γ, IL-10, and IL-6) in the sera of the transplanted mice. Significant levels of human IL-10 (between 23 and 605 pg/ml) but not IL-2, IL-6, and IFN-γ (less than 5 pg/ml) were detected using an ELISA in the serum of lymphoma-bearing mice (data not shown).

**Histological and Molecular Analyses.** Mice that died from malignant lymphoma and long-term surviving animals (>day 100) were subjected to histological analyses. As shown in Fig. 4A, liver sections showed infiltration with large-cell anaplastic lymphomas (Fig. 4C) containing multiple mitoses. The diagnosis of B-cell anaplastic lymphoma was confirmed by immunohistological staining using anti-B-cell antibodies (CD19 and CD20) on snap-frozen tissue sections. Histological analysis of liver, spleen, kidney, and lung specimens from long-term surviving mice showed no tumor infiltration (Fig. 4B). PCR analysis revealed the presence of EBV-specific DNA in tumor-infiltrated specimens but not in long-term surviving mice (Fig. 5). To determine whether residual human T cells remained in surviving mice, spleen sections were analyzed for the presence of rearranged human TCR-γ chain genes (Fig. 6). Multiple DNA fragments of approximately 200 bp were generated by PCRs with oligonucleotide primers specific for human TCR-γ gene segments indicating the presence of human T cells with polyclonally rearranged TCR-γ chains in the biopsy. Control specimens from mice without human cell inoculation did not contain the human TCR-γ gene as measured by identical PCR analysis (compare Fig. 6, Lane 2).

**DISCUSSION**

Bispecific antibodies plus CD28 antibodies have been shown to activate T cells to secrete cytokines and lyse the tumor cells in the presence of autologous lymphoma cells in vitro (11, 16). This antibody combination was also effective in inhibiting the growth of human lymphoma cells upon PBMC transplantation into SCID mice. Results presented here demonstrate that bispecific antibodies can also prevent development of high-grade EBV-positive B-cell lymphomas in immunocompromised SCID mice. Histological analysis revealed infiltration of large anaplastic B cells in lymphoid organs and PCR experiments showed the presence of EBV in these tumors. It has been suggested that EBV-positive B-cell lymphomas develop in immunocompromised hosts (AIDS patients, posttransplantation) due to ineffective T-cell control. Inhibitory cytokines produced by the lymphoma cells such as IL-10, which has also been detected in lymphoma-bearing SCID mice, may add to this suppressive effect. In fact, we and
others have shown that EBV can induce IL-10 production in Burkitt’s lymphoma cells (21).

Furthermore, the analysis with defined mismatched bispecific antibodies demonstrates clearly that the capacity to redirect T-cell action toward a given target will not be affected by preparations of bi-isotypic antibodies harboring light chain mismatches.

To determine whether treatment with bispecific antibodies induces an immune response, surviving mice were rechallenged with autologous EBV-induced B cells. In this experiment, the rechallenged mice were not treated with antibodies. Although naive age-matched control animals given injections of EBV-transformed B cells died from tumor within 40 days, the rechallenged animals did not develop tumor until 80 days posttransplantation (data not shown). Experiments to isolate and characterize the putative memory T cells in these animals have to be performed to prove this hypothesis.

It has been shown in vitro that CD3 × antitumor bispecific antibodies are not a sufficient stimulus to activate resting T cells and that a second signal, which can be delivered by monospecific bivalent CD28 antibodies, is also required. However, we found no significant difference between the ability of CD3xCD19 bispecific antibodies alone or in combination with CD28 antibodies to protect mice from tumor growth. This apparent discrepancy could be due to the high expression of the costimulatory molecule, i.e., the B7–1 molecules by the EBV-transformed tumoral B cells in vivo, given that blocking B7–1 antibodies show a nearly complete abrogation of the capacity of EBV-transformed B cells to deliver costimulatory signals in vitro. Blocking experiments with B7–1 and B7–2 monoclonal antibodies in SCID mice are currently underway in our laboratory to demonstrate the need for CD28 costimulation in B7-positive lymphomas.

EBV has been linked to a number of hematological malignancies, especially in immunocompromised hosts, and can be detected in Burkitt’s lymphoma, Hodgkin’s disease, nasopharyngeal carcinoma, and in a group of immunoblastic lymphomas in allograft recipients and patients infected with the HIV (22). Although the pathogenesis of these lymphoproliferative disorders is poorly understood, it is likely that the lack of T-cell control results in the development of EBV-positive tumors. Our results are consistent with this hypothesis and demonstrate that activation of autologous T cells can inhibit lymphomagenesis. We have demonstrated that T cells can be directed against EBV-positive human B-cell lymphomas by treatment with bispecific antibodies. Similar findings have been reported by Papadopoulos et al. (23) and Mackinnon et al. (24), who demonstrated that donor leukocyte infusions were an effective treatment in EBV-associated lymphoproliferative disease arising after allogenic bone marrow transplantation from donor B cells.

Our findings may also have implications for the development of tumor vaccines for clinical studies. Although tumor vaccines have been produced from cell lysates or extracts from autologous tumor cells mixed with chemical adjuvants, tumor-specific responses could only be demonstrated in a few cases. Active tumor vaccination studies were usually not effective. Our method of targeting T cells to tumor cells may offer an alternative vaccination strategy against autologous tumors, circumventing the disadvantages of individual vaccine preparation or manipulation.

ACKNOWLEDGMENTS

We thank R. Hippler-Altenburg and S. Tawadros for excellent technical assistance, H. Hoogenboom and E. Jurkiewicz for critical reading of the manuscript, and Behring (Köln, Germany), Life Technologies, Inc. (Karlsruhe, Germany), and Biermann for technical help. We also thank Klosterfrau (Köln, Germany) for their generous help.
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