Cytolysis of Leukemic B-Cells by T-Cells Activated via Two Bispecific Antibodies

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ABSTRACT

Bispecific monoclonal antibodies can be used in the activation of effector cells to lyse autologous tumor cells. We analyzed the activation of human T-cells in vitro with bispecific monoclonal antibodies, which were generated by hybridoma-hybridoma fusion. Pretreated autologous and autologous T-cells could be triggered to lyse tumoral B-cells in the presence of CD3 × CD19 bispecific antibodies. In addition, the combined use of two CD3 × CD19 plus CD28 × CD22 bispecific antibodies induced optimal interleukin 2 secretion by Jurkat T-cell acute lymphocytic leukemia cells in the presence of target B-cells. The same antibody combination was able to generate cytolytic effector cells without prior activation, when resting T-cells were cocultured with freshly isolated autologous leukemic B-cells in the presence of the bispecific antibodies. The results suggest that signals required to activate cytolytic T-cell precursors can be provided by the two bispecific antibodies. Although activation of resting T-cells can be achieved by CD3 × CD19 bispecific antibodies in association with monospecific bivalent CD28 antibodies, the second bispecific antibody, CD28 × CD22, further increases the specificity of the target cell dependent activation of T-cells. When used for immunotherapy of B-cell malignancies, the CD3 × CD19 and CD28 × CD22 bispecific antibody combination may avoid the need for ex vivo activated effector cells, because the antibodies may induce T-cell activation directly at the tumor site.

INTRODUCTION

A major requirement in the immunotherapy of malignant tumors is the activation of autologous T-cells. Many attempts have been made to focus on autologous T-cells in tumor therapy. Some of these studies showed that monovalent bispecific antibodies could serve as a unique tool in the targeting of activated T-cells to the tumor (1–4). Bispecific antibodies recognizing the TCR/CD33 complex and an epitope on the tumor cell can induce preactivated T-cells to lyse the tumor cells (5). Clinical studies by Nitta et al. (6) reported complete responses in 4 of 10 patients with malignant glioma after intrathecal application of CD3 × anti-glioma bispecific antibodies in combination with LAK cells. However, the therapy with LAK cells has several disadvantages and is frequently associated with severe side effects. The use of the two bispecific antibodies may avoid the requirement of LAK cells and ex vivo activation of T-cells. The benefit to use bispecific antibodies is the activation of T-cells at the tumor site. Since resting T-cells cannot be activated alone by the cross-linking of their TCR/CD3-protein complex a second signal is required (7). As suggested by Jung et al. (8) this second signal can be provided by cross-linking of the CD28 homodimer (9, 10).

In the present study the activation of resting T-cells by monoclonal bispecific antibodies was analyzed in more detail. Cells of the Jurkat T-cell acute lymphocytic leukemia line, used as a model of resting T-cells (11, 12), were stimulated with two bispecific monoclonal antibodies, CD3 × CD19 and CD28 × CD22. The optimal activation of Jurkat T-cells was observed after coculture with both bispecific monoclonal antibodies and CD19, CD22 double positive B-cells. In addition, T-cells from patients with follicular lymphoma could be activated by the bispecific antibodies with subsequent cytolysis of autologous tumoral B-cells.

The activation of resting T-cell could also be achieved with CD3 × CD19 bispecific in combination with monospecific bivalent CD28 antibodies. However, targeting and activation of T-cells with two bispecific antibodies may further increase the tumor specificity of this therapeutic approach.

MATERIALS AND METHODS

Cell Lines and Isolation of Hybrid-Hybridoma. The hybridoma OKT3 (CD3/IgG2a; American Type Culture Collection, Rockville, MD) and the hybridoma 6A4 (CD19/IgG1; R. Levy, Stanford, CA) were kindly provided by Dr. K. Thielemanns (University of Brussels, Brussels, Belgium). The hybridoma 15E8 (CD28/IgG1) was kindly provided by Dr. R van Lier (Netherlands Center for Blood Transfusion, Amsterdam, the Netherlands). The T-cell clones SC, TB, and HaCD3+, CD8+, TCRαβ+ were derived from patients with allergic skin lesions and were a gift of Dr. M. Hertel (Hautklinik, Universität Köln, Köln, Germany). The Epstein-Barr virus transformed B-cell line LAZ509 (CD19+, CD20+, CD22+, CD50+, BB1+) was kindly provided by Dr. S. Meuer (DKFZ, Heidelberg, Germany). All hybridoma cell lines were grown in RPMI (Gibco, Egggenstein, Germany) medium supplemented with l-glutamine (2 mM), penicillin (100 IU), streptomycin (100 μg/ml), and 1% fetal calf serum (referred as complete medium A). The isolation of the CD3 × anti-L-D1 antibodies, capable of retargetting T-cells to colon carcinoma cells, have been described elsewhere (18).

OKT3 hybridoma cells and 15E8 were selected for HGPRT deficiency by culture in s-azaguanine (20 μg/ml) containing medium. Before cell fusion, the second fusion partner, 6A4 or HD239 (IgG2a, CD22), was pretreated with a lethal dose of iodoacetamide (5 mM; 15 min, 4°C). Cell fusion was carried out using a standard protocol (13). Briefly, 3 × 103 cells of each parental line were washed twice in RPMI, mixed, and subsequently fused by addition of prewarmed polyethylene glycol 1500 (1 ml; Boehringer, Germany).

Hybrid-hybridoma were preselected in complete medium A supplemented with HAT. Supernatants were tested in an ELISA for the presence of both isotypes in individual wells. Multiple subcloning experiments were performed to establish stable antibody producing clones.

Double Isotype ELISA. Goat anti-mouse IgG2a antibodies (1 μg/ml; PBS; Southern Biotechnology Associates, Birmingham, United Kingdom/DUNN, Zuerihen, Germany) were coated onto microtiter plates (Nunc, Roskilde, Denmark). The plates were blocked with 200 μl 1% nonfat milk/PBS for 1 h at room temperature and washed twice with 0.9% NaClO/0.1% Tween. Culture supernatants were added and incubated for 1 h. Subsequently horseradish peroxidase labeled goat anti-mouse IgG1 antibodies (1/4000; SBA) were added. Bound antibodies were finally detected by addition of the substrate ABTS (2,2'-azino-di-[3-ethylbenzthiazolin-sulphonate (6)] (Boehringer). The extinction at 405 nm was measured with an ELISA reader. (Dynatech, Chantilly, VA).

Culture supernatants, containing bispecific antibodies, was diluted with 2 mM NH4SO4 to a final concentration of 0.85 mM NH4SO4, loaded on a phenyl-Sepharose (Pharmacia, Freiburg, Germany) column and eluted by a continuous gradient from 0.85 mM NH4SO4/0.1 mM Na2HPO4, pH 7.2, to 0.001 mM NH4SO4/0.1 mM Na2HPO4, pH 7.2.
Induction of Cytotoxic T-cells. The generation of cytotoxic T-cells has been described elsewhere (14). Briefly, Ficoll separated PBMCs from healthy donors were cultured in complete medium A supplemented with 5 x 10^-5 M 2-mercaptoethanol and PHA (Wellcome, Germany) (1 μg/ml) for 48 h. Subsequently the cells were harvested, washed, and resuspended in complete medium A supplemented with recombinant IL2 (30 IU/ml; Cetus). After 8 days of culture the cells were used in the cytotoxicity assay.

Cytotoxicity Assay. LAZ509 B-cells (5 x 10^6) were incubated for 1 h at 37°C with 100 μCi Na^25CrO_4 (3 mCi/ml; Medgenics, Düsseldorf, Germany). The cells were washed twice in RPMI and resuspended in complete medium A (1 x 10^6 cells/ml). K562 cells (1 x 10^6) were labeled with europium diethylene-triaminepentaacetic acid as described (15). Target cells (100 μl/well) were plated in V-shaped 96-well plates and the indicated amounts of effector cells and antibodies were added. The plates were centrifuged for 3 min at 250 x g and incubated for 3-4 h at 37°C in a 7% CO_2 atmosphere. Bystander lysis was measured in a time resolved fluorometry (Wallac, Finland). The specific 51Cr release was calculated as

\[
\% \text{ of specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

T-Cell Stimulation. Goat anti-mouse IgG1 antibodies (1 μg/ml PBS; SBA) were coated overnight onto 96-well plates. The plates were washed and blocked with RPMI/10% fetal calf serum (200 μl/well), and serial dilutions of culture supernatants were added and incubated for 1 h at room temperature. Plates were washed and subsequently PHA preactivated (5 μg/ml, 48 h) PBMCs from healthy donors (1 x 10^5 cells/well) were added. T-Cell proliferation was measured by [3H]thymidine incorporation (0.5 μCi/well; Amersham, Braunschweig, Germany) during the last 16 h of the total culture period of 72 h. The data were given as the mean cpm ± SEM of triplicate measurements. or europium release was calculated as

\[
\% \text{ of specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximum release} - \text{spontaneous release}} \times 100
\]

Immunofluorescence Staining. LAZ509 B-cells and Jurkat T-cells (1 x 10^5 cells/test) were incubated with 10-fold diluted culture supernatants for 1 h at 4°C. The cells were then washed and stained with isotype specific FITC labeled goat anti-mouse IgG. Staining was evaluated by FACScan (Becton Dickinson, Heidelberg, Germany). A minimum of 1 x 10^4 cells was analyzed using the FACScan software. Staining buffer (PBS/1% bovine serum albumin/0.01% NaN_3) was used for all fluorescence activated cell sorting experiments. Costimulation Experiments with Jurkat T-cells and LAZ509 B-cells. LAZ509 cells (1 x 10^5) were incubated with supernatants of the indicated cell lines for 1 h at 4°C. The cells were washed twice in complete medium A to remove unbound antibodies, and subsequently Jurkat cells (1 x 10^5/ml) were added (1 ml). After gentle centrifugation (200 x g, 2 min) the cells were incubated for 24 h at 37°C in 7% CO_2. The supernatants were then harvested.
were tested in two independent assays. Deficient hybridoma line OKT3 (CD3, IgG2a) and iodoacetamide (Beckmann, München, Germany). All data are expressed as the mean cpm ± SEM of triplicate measurements. Bars, SEM.

and tested for IL2 activity using the murine T-cell line CTLL (16). Briefly, 2 × 10^5 CTLL-cells were plated in 96-well plates in the presence of culture supernatants or serial dilutions of recombinant IL2. Proliferation of CTLL cells was quantified by [³H]thymidine incorporation (0.5 μCi/well) during the last 8 h of the 48-h culture period and measured with a liquid scintillation counter (Beckman, München, Germany). All data are expressed as the mean cpm ± SEM of triplicate measurements.

RESULTS

Isolation and Functional Analysis of CD3 × CD19 Bispecific Monoclonal Antibodies. The bispecific monoclonal antibody OKT3 × 6A4 (CD3 × CD19) was obtained from a fusion of the HGPRT deficient hybridoma line OKT3 (CD3, IgG2a) and iodoacetamide treated 6A4 hybridoma cells (CD19, IgGl). The secreted antibodies were tested in two independent assays.

OKT3 × 6A4 bispecific antibodies were functionally tested by stimulation of PHA preactivated PBMC from healthy donors. Goat anti-mouse IgGl coated plates were incubated either with OKT3 × 6A4 (CD3 × CD19) bispecific antibodies or with the two parental antibodies OKT3 or 6A4 as controls. As shown in Fig. 1A, OKT3 × 6A4 induced proliferation of preactivated peripheral T-cells. The parental antibodies alone or in combination did not induce T-cell proliferation. The capacity of bivalent CD3 antibodies to stimulate T-cells was determined on plates coated with goat anti-mouse IgG2a antibodies. The results obtained from these experiments indicate that the stimulation induced by the bispecific CD3 × CD19 antibodies is comparable to the T-cell stimulation mediated by cross-linked bivalent CD3 antibodies (Fig. 1B). In order to evaluate the activation potential of the purified CD3 × CD19 bispecific antibodies, these antibodies were used in comparison to bivalent parental CD3 antibodies. Results shown in Fig. 1B indicate that the stimulation by bivalent CD3 antibodies was as good as the stimulation mediated by bispecific antibodies.

Cytolysis of Leukemic B-Cells by Preactivated T-Cells. OKT3 × 6A4 antibodies were tested for their ability to mediate B-cell target lysis. Effector T-cells from healthy donors generated by stimulation with PHA and IL2 (12) as well as CD3+, CD8+ T-cell clones could lyse allogeneic B-cells (LAZ509) in the presence of CD3 × CD19 bispecific antibodies (Fig. 2). Even at a concentration of 4 ng/ml CD3 × CD19 the bispecific antibodies led to specific target cell lysis. In control experiments the parental antibodies OKT3 and 6A4 did not induce significant cell lysis under the same conditions. The effects of the bispecific antibodies upon CD19 negative cells can be seen in Fig. 3. In this experiment K562 and LAZ509 cells were coincubated together with cytotoxic T-lymphocytes and the indicated purified antibodies. There was no lysis of K562 cells in the presence of CD3 × CD19 bispecific antibodies. However, there was substantial killing of K562 when parental CD3 antibodies or crude culture supernatant of the CD3 × CD19 or CD3 × anti-L-D1 antibodies were used. This effect is probably due to the presence of bivalent CD3 antibodies which have a high affinity to the Fc receptors on K562 cells. In addition, there was no cytolysis of the B-cell target LAZ509 in the presence of CD3 × anti-L-D1 bispecific antibodies.

Isolation and Characterization of the CD22 × CD28 Bispecific Antibodies. The bispecific monoclonal antibody CD22 × CD28 is secreted by the hybrid-hybridoma cell line 15E8 × HD239. This cell line was obtained by cell fusion of the HGPRT deficient mutant 15E8-H (CD28, IgGl) with iodoacetamide treated cells of the hybridoma HD239 (CD22, IgG2b). The supernatants were initially tested for the presence of both IgGl and IgG2b isotypes by ELISA in the same wells.
ACTIVATION OF T-CELLS VIA TWO BISPHERIE ANTIbODIES

Table 2 Stimulation of Jurkat T-cells with bispecific monoclonal antibodies

<table>
<thead>
<tr>
<th>mAbα</th>
<th>Specificity</th>
<th>IL2β (IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OKT3 + 6A4 + 15E8 + HD239</td>
<td>CD3, CD19, CD28, CD22</td>
<td>0.6 ± 0.09</td>
</tr>
<tr>
<td>15E8 × HD239</td>
<td>CD28 × CD22</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>OKT3 × 6A4</td>
<td>CD3 × CD19</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>OKT3 × 6A4 + 15E8 × HD239</td>
<td>CD3 × CD19, CD28 × CD22</td>
<td>22.1 ± 1.2</td>
</tr>
<tr>
<td>OKT3 × 6A4 + PMAα</td>
<td>CD3 × CD19</td>
<td>19.2 ± 0.4</td>
</tr>
</tbody>
</table>

α Monoclonal antibodies (mAb) were preincubated with LAZ509 B-cells (1 × 10^5 cells/test) for 1 h at 4°C. The cells were washed twice and cocultured with 1 × 10^6 Jurkat cells for 24 h. 
β Supernatants from each individual culture were tested for IL2 activity in a standard CTL assay. 
α PMA, phorbol myristate acetate.

Culture supernatants containing both IgG1 and IgG2b isotypes were further examined for binding to Jurkat T-cells (CD28+, CD22+) and LAZ509 B-cells (CD28-, CD22-), respectively. As shown in Fig. 4, binding of bispecific antibodies 15E8 × HD239 to LAZ509 cells was demonstrated with FITC labeled goat anti-mouse IgG1 antibodies. Binding of the 15E8 × HD239 bispecific antibodies to Jurkat T-cells was demonstrated with FITC labeled goat anti-mouse IgG2b antibodies. These results prove that the 15E8 × HD239 (CD22 × CD28) bispecific antibodies possess two functional binding sites.

**Cytolysis of Leukemic B-Cells by Antologous Cytotoxic T-Cells Generated by Stimulation with CD3 × CD19 and CD28 × CD22 Bispecific Antibodies.** Cytolytic effector T-cells were generated by coculture of freshly isolated, monocyte depleted PBMC with autologous leukemic B-cells from a patient with follicular lymphoma and the combination of the two bispecific antibodies. After an initial stimulation period of 8 days the T-cells were harvested and tested in a standard cytotoxicity assay with 51Cr-labeled target B-cells. It was shown that these activated T-cells could lyse the leukemic B-cells in the presence of CD19 × CD3 bispecific antibodies (Fig. 3). The parental antibodies did not induce T-cell activation and furthermore there was no cytotoxic activity against the NK-cell target cell K562 (Table 1).

**Activation of Jurkat T-Cells.** The activation of resting T-cells was studied using Jurkat T-cells. Jurkat T-cells were induced to secrete optimal amounts of IL2 when cultured with the two bispecific antibodies, OKT3 × 6A4 and 15E8 × HD239, in the presence of B-cells as a cross-linking surface (Table 2). Cross-linked CD28 antibodies with soluble monospecific bivalent CD28 antibodies can induce Jurkat cells to secrete IL2. To rule out the effects of the CD28 × CD22 bispecific antibody, the B-cell targets were preincubated with the indicated antibodies and washed extensively to remove unbound CD28 antibodies. No augmentation of IL2 production was seen when monospecific bivalent CD28 antibodies were preincubated with the LAZ509 B-cells, suggesting the absence of functional Fc receptors. Optimal stimulation of Jurkat cells was induced by OKT3 × 6A4 antibodies with phorbol myristate acetate. The parental antibodies as well as 238T28 bispecific antibodies or phorbol myristate acetate alone did not induce IL2 secretion. The effects caused by stimulation with OKT3 × 6A4 antibodies alone were approximately 30% of the maximum IL2 secretion and may be due to the expression of the B7 antigen on LAZ509 B-cells, the natural ligand for CD28. There was also no stimulation when Jurkat T-cells were incubated with the bispecific antibodies (CD3 × CD19, CD22 × CD28) in the absence of B-cells (data not shown).

**DISCUSSION**

Most therapeutic strategies using bispecific antibodies have been focused on antibodies that recognize the CD3 molecule, expressed by nearly all mature T-cells, and tumor specific or tumor associated antigens (17, 18). As has been shown in experimental tumor models like the murine 38C13 B-lymphoma, CD3 × anti-idiotypic antibodies can be used effectively for immunotherapy of B-cell lymphomas (19). However, there is evidence that only preactivated T-cells are recruited for tumor cell lysis by such CD3 × anti-tumor bispecific antibodies. Thus, immunotherapy with the CD3 × anti-tumor antibodies alone requires *ex vivo* T-cell activation.

The activation of effector T-cells *in vivo* through bispecific antibodies may avoid the additional requirement for LAK cells. Most T-lymphocytes in the peripheral blood or at the tumor site are in a resting state and can be activated only after cross-linking of their CD3/TCR complex in combination with a second signal.

In this study we present evidence for an effective and specific activation of resting T-cells by two bispecific antibodies. Furthermore it could be shown that the T-cell activation resulted in a drastic increase of the cytolytic activity. The OKT3 × 6A4 bispecific antibodies (CD3 × CD19) recognize an epitope on the e-chain of the CD3 molecule on T-cells and the CD19 antigen present on B-cells, while the bispecific antibodies 15E8 × HD239 (CD28 × CD22) bind the CD28 epitope on T-cells and the CD22 antigen on B-cells. The approach with these two bispecific antibodies may allow the activation of T-cells in patients with B-cell tumors, which carry both CD19 and CD22 antigens. The cross-linking of both CD3 and the CD28 antigens results in activation of T-cells (Table 2). Since bispecific antibodies are monovalent for each antigen, T-cell activation occurs only in the presence of CD19/CD22 double positive B-cells. Jurkat T-cells could be activated with subsequent IL2 secretion when cocultured with the Epstein-Barr virus transformed B-cell line LAZ509 and the bispecific antibodies CD3 × CD22(OKT3 × 6A4) and CD28 × CD22(15E8 × HD239) (Table 2).

The results may have further implications for the therapy of malignant B-cell tumors. A therapy with CD3 × CD19 and CD28 × anti-common idiotype antibodies may lead to the activation of tumor infiltrating lymphocytes and perhaps idiotype specific T-cells (20–22). These T-cells, which are supposed to exist according to the idiotype network theory, may be the ideal target T-cell population for this therapy (23, 24). Initially activated by this stimulation the T-cells could further be expanded by stimulation with their natural antigen (idiotope determinant), presented by the tumoral B-cell.

Although LAZ509 cells are positive for the B7 antigen, the natural ligand for the CD28 homodimer on T-cells (25, 26), optimal stimulation required both OKT3 × 6A4 (CD3 × CD19) and 15E8 × HD239 (CD28 × CD22) antibodies. This phenomenon was also seen with a variety of B-cell tumors (27). None of the examined B-cells tumors, although positive for the B7 antigen, could costimulate T-cells in the presence of CD3 × CD19 bispecific antibodies. The usage of activated normal B-cells, however, did not require the signaling via CD28 antibodies (data not shown). The lack of the intercellular B7-CD28 costimulation is not clear yet and may be due to either a defect in the B7 molecule expressed on tumor B-cells or to inhibitory signals like IL10 given by the tumoral B-cells.

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