Bispecific Antibody-mediated Target Cell-specific Costimulation of Resting T Cells via CD5 and CD28

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ABSTRACT

Induction of T-cell activation requires multiple signals provided by cell surface receptor interactions and/or cytokines. T-cell stimulation via the T-cell receptor/CD3 complex provides an important initial activation event which, when combined with the proper costimulatory signals, results in an activated effector T cell. In this report, we have investigated the effectiveness of epithelial glycoprotein-2 (EGP-2) positive tumor target cells to induce specific T-cell stimulation via CD3, CD5, and CD28 using various combinations of bispecific monoclonal antibodies (BsMab) directed against CD3, CD5, or CD28 alone on the one hand and the pan-carcinoma-associated antigen EGP-2 on the other. Induction of T-cell activation was investigated by assessment of CD69 expression, induction of proliferation, and acquisition of cytolytic potential. EGP-2-specific induction of T-cell activation was observed using combinations of BsMab which simultaneously ligated CD3/CD5, CD3/CD28, or CD3/CD5/CD28 with EGP-2. Activation with CD3-, CD5-, or CD28-based BsMab alone did not result in significant induction of T-cell activation in the presence or absence of EGP-2-positive target cells. Simultaneous ligation via CD5/CD28 resulted in partial T-cell activation, including CD69 up-regulation and increased cytolytic activity. Stimulation via CD3 and CD5 or CD28 could be further increased by the addition of exogenously added recombinant Interleukin 2. In contrast, T-cell activation by simultaneous ligation of CD3/CD5/CD28 could not be further augmented by addition of exogenous interleukin 2, indicating that T-cell activation via the combination of CD3, CD5, and CD28 results in complete T-cell activation. Our results show that rapid and target cell-specific induction of T cells is possible using combinations of BsMab directed against different costimulatory molecules. Simultaneous costimulation via CD3/CD5/CD28 results in the most complete activation of T cells.

INTRODUCTION

CTL efficiently kill opposing target cells upon TCR-mediated recognition of the target cell. BsMab containing a CD3 or TCR specificity are able to redirect the lethal hit of CTL against any target cell surface antigen and can mediate in an MHC-unrestricted killing of tumor cells by CTL (1, 2). Efficient BsMab-mediated killing of tumor cells by activated CTL has been shown in vitro and in vivo. Preactivation of the effector T cells is an important prerequisite for the establishment of effective antitumor reactions (3–6). In vitro preactivation of T cells is possible by relatively simple procedures, such as incubation of isolated PBMC with a mitogenic antibody directed against the CD3 complex (4, 7). T-cell activation, in this case, is based on the cross-linking of CD3 molecules on the T cell by anti-CD3 antibodies immobilized on monocyes. The presence of activated monocytes is indispensable in this concept (8–10), which might be the reason for the disappointing results with regard to T-cell activation in vivo using CD3-recognizing antibodies such as OKT-3 (9, 11, 12). Immune activation in human cancer patients can be achieved by the application of recombinant IL-2. Although T-cell activation is induced by IL-2 therapy (13), serious toxic side effects accompany IL-2 treatment, which has been a major drawback for its use in an anticancer treatment modality.

Activation of T cells can be regarded as the result of an array of stimulatory signals in which divergent cell surface molecules such as CD3, CD2, CD28, and CD5, as well as various adhesion molecules and cytokine receptors, may be involved (3, 14, 15). It is well established that costimulation via CD28 besides TCR-CD3 activation results in a rapid activation of resting T lymphocytes (16–18). In fact, various preclinical in vitro and in vivo immunotherapeutical treatment protocols have been described that involve costimulatory signaling of T cells via CD28 (19–22). Vaccination protocols with tumor cells, transfected with the CD28 ligand B7, also seem successful, not only with regard to the eradication of the transfected tumor cells, but also induce an effective T cell-based immune response against B7-negative tumor cells (23). Also, BsMab directed against the CD28 receptor and a tumor-associated antigen, in combination with a CD3-containing BsMab, have been shown to provide essential in vivo costimulatory T-cell signaling, resulting in the in vivo eradication of tumor and the establishment of a memory response against subsequent challenges with the same tumor (19, 21). Also, CD5 might be useful for delivering costimulatory signals. The CD5 molecule is present on nearly all peripheral T cells, is expressed in close conjunction with the TCR/CD3 complex, and has been shown to increase intracellular calcium in T cells upon its cross-linking (24, 25). Together with lectins or anti-CD3 antibodies, anti-CD5 mAbs have been shown to augment and sustain T-cell proliferation (26). Furthermore, induction of expression of early activation markers preferentially on naïve T cells has been described as a result of costimulation via CD5 (18). The use of combinations of BsMab, for instance a CD3 containing BsMab, together with BsMab providing a costimulatory signals, seems an attractive approach for the induction of a cytotytic T-effector cell population for the following reasons: (a) systemic T-cell activation, as induced by systemic application of IL-2, might become dispensable since T-cell activation involving some of the costimulatory activation pathways renders the CTL independent of exogenous IL-2; (b) T-cell activation occurs only at the site of the tumor, which might minimize unwanted toxicity from systemic immune activation; and (c) multiple costimulatory pathways, that are ignored with IL-2 treatment, can be combined to induce optimal T-cell activation.

To be able to select ideal sets of activation signals, experience is needed on how these stimulatory pathways are involved and interact with each other in the activation of T cells in clinically relevant settings. In this report, we describe the effects of BsMab-mediated, tumor-specific T-cell activation through combinations of BsMab directed against CD3, CD5, and CD28 on the one hand and the tumor-associated antigen EGP-2 at the other hand. EGP-2 is a M, 38,000 pancarcinoma-associated transmembrane glycoprotein that has been described as a target antigen for site-directed immunotherapy in a number of clinical settings, including BsMab (27–29). T-cell activation was studied using whole-blood culture assays which better mimic...
the in vivo situation since monocyte activation, as a result of the normally used isolation procedures, does not occur (9, 10, 30). Our results indicate that, in the presence of EGP-2-positive target cells, complete IL-2-independent target cell-specific activation can be induced in resting peripheral blood T lymphocytes.

MATERIALS AND METHODS

**Cell Lines.** GLC-1 (EGP-2 negative) and a clonal derivative thereof, GLC-1M13 (EGP-2 positive), are human SCLC-derived cell lines (31). The cell lines were cultured according to routine procedures in complete medium, i.e., RPMI 1640 (GIBCO-BRL, Paisley, United Kingdom) supplemented with 14% heat-inactivated FCS (GIBCO-BRL), 2 mM glutamine (GIBCO-BRL), 60 µg/ml gentamicin (GIBCO-BRL), 0.05 mM β-mercaptoethanol (Merck, Darmstadt, Germany), and 1 mM sodium pyruvate (GIBCO-BRL) at 37°C in a humidified atmosphere containing 5% CO2.

**Lymphocyte Isolation.** PBMC were obtained from heparinized peripheral blood. Isolation was done by density centrifugation of diluted (1:1 PBS) blood on Lymphoprep (Nycomed, Oslo, Norway) at 2400 rpm for 20 min. The PBMC fraction was washed twice by resuspension in RPMI 1640 and centrifugation at 1800 rpm (first time) and 1200 rpm (second time) for 10 min. After isolation, PBMC were collected in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated, human pooled serum, 2 mM glutamine, and 60 µg/ml gentamicin.

**CTL Activation for Analysis of Cytolytic Activity.** To obtain activated cytotoxic effector T cells, isolated PBMC were incubated at a concentration of 0.5 × 10^6 cells/ml in bulk culture for 3 days in complete medium supplemented with 5% culture supernatant of the mitogenic anti-CD3 mAb WT-32 (Ref. 32; giving about 0.5 µg IgG/ml end concentration), followed by washing RPMI 1640 and incubation for 2 additional days at a concentration of 0.5 × 10^6 cells/ml in complete medium supplemented with 100 IU/ml IL-2 (EuroCetus, Amsterdam, the Netherlands). For investigation of the cytolytic activity of T cells after BsMab-mediated activation, freshly isolated PBMC were used which monocytes had been removed by 2 rounds of plastic adherence. Monocyte-depleted PBMC (5 × 10^6) were incubated for 24 h in 100 µl complete medium supplemented with the indicated BsMab in the presence of 2.5 × 10^4 irradiated (45 Gy total dose, 6 Gy/min) GLC-1M13 or GLC-1 cells and 100 IU/ml IL-2 or not in 96-well microtiter culture plates (Costar, Cambridge, MA). After incubation for 24 h, fresh 51Cr-labeled target cells were added as indicated below, and cytolytic potential of the CTL was assessed.

**CTL Activation for Flow Cytometry and Proliferation Analysis.** T-cell activation to be analyzed by flow cytometry or [3H]thymidine incorporation was done using whole-blood cultures. To this end, heparinized whole blood was collected from healthy volunteers and diluted 1:10 with RPMI 1640 containing irradiated (45 Gy total dose, 6 Gy/min) GLC-1M13 or GLC-1 cells, the indicated BsMab, and IL-2 or not. For flow cytometric analysis, 0.5 ml of the diluted whole-blood cell suspension containing 1 × 10^7 target cells, 0.1 µg/ml each of the BsMab, and 100 IU/ml IL-2 or not was pipetted into a sterile culture tube (Greiner, Frickenhausen, Germany) and incubated for 24 h at 37°C, 5% CO2. After incubation for 24 h, expression of CD69 on CD4- and CD8-lymphocytes was assessed by flow cytometry as described below. For analysis of proliferation, 0.1 ml diluted whole-blood cell suspension containing 2 × 10^7 target cells, 0.1 µg/ml each of the BsMab, and 100 IU/ml IL-2 or not was pipetted into a 96-well microtiter plate and incubated for 72 h followed by assessing the proliferation as described below.

**Bispecific Antibodies.** The following BsMab were used in the study: BIS-1, directed against CD3 and EGP-2; BIS-18, directed against CD5 and EGP-2; and BIS-20, directed against CD28 and EGP-2. All BsMab were produced by hybrid hybridoma cell lines (Quadroma), generated by fusion of two hybridoma cell lines, as described (28, 33). Quadroma BIS-1 was generated by fusion of the hybridomas RIV-9 (anti-CD3, IgG3; a kind gift of Dr. H. Kreeftenberg, RIVM, Bilthoven, the Netherlands) and MOC-31 (anti-EGP-2, IgG1; Ref. 34). Quadroma BIS-18 was generated by fusion of the hybridomas 83-P2E6 (anti-CD5, IgG2b; MCA development, Groningen, the Netherlands) and MOC-31. Quadroma BIS-20 was generated by fusion of the hybridomas CLB-CD28 (anti-CD28, IgG1; Ref. 35) and MOC-181 (anti-EGP-2, IgG2a). BsMab production by the quadroma cell lines was done in a hollow fiber culture system (Technomara, Zurich, Switzerland). Purification of BIS-1 from other nonbspecific antibodies also produced by the quadroma was done by protein A column chromatography as described (28). Also, BIS-18 was purified by loading crude BIS-18 IgG containing supernatant onto a protein A column (Pharmacia, Uppsala, Sweden) at pH 7 and eluting the different antibody fractions by lowering the pH gradually. BIS-20 was purified by cation-exchange column chromatography. Protein A-purified BIS-20-IgG was dialyzed against 20 mM Bis-Tris (pH 6.8) and loaded onto a Mono-S cation exchange column (Pharmacia). The different antibody fractions were eluted using a buffer gradient 20 mM–0.2 M Bis-Tris (pH 6.8). After purification, all BsMab were dialyzed against PBS and stored sterile at a concentration of 30–100 µg/ml at –20°C. The purified BsMab were all tested for their bispecific characteristics by immunostaining against EGP-2-positive tumor cells and T cells followed by flow cytometric analysis.

**Antibodies Used for Staining.** For flow cytometric analysis, the following antibodies and conjugates were used. Anti-CD69-FTTC, anti-CD4-PE, and anti-CD8-biotin and streptavidin-phycocyanin were obtained from Becton Dickinson (Mountainview, CA). Detection and characterization of BsMab were done using isotype-specific immun conjugates, i.e., FITC- or PE-labeled goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology, Inc., Birmingham, AL).

**Flow Cytometry.** BsMab-induced CD69 expression by T cells was analyzed by incubation of 100 µl whole-blood cell suspensions, activated as described above, with 10 μl of each of the immunon conjugates anti-CD4-PE or anti-CD8-biotin and anti-CD69-FTTC at 4°C for 30 min. After one wash with ice-cold PBS, anti-CD8-biotin-stained samples were incubated with 10 μl streptavidin-phycocyanin in 50 μl PBS at 4°C for 30 min. Control stainings included single stainings with anti-CD4-PE, anti-CD8-biotin/streptavidin-phycocyanin, and anti-CD69-FTTC. After staining, cell samples were fixed, and erythrocytes were lysed with 2 ml FACSLyse solution at room temperature for 15 min, followed by centrifugation at 2500 rpm for 2 min and washing of the cell pellet with PBS. After centrifugation, cells were resuspended in 150 µl PBS and analyzed on a Coulter ELITE cytometer (Coulter Electronics, Hialeah, FL) using an argon laser (488 nm) for FTTC and PE excitation.

**Proliferation Assays.** BsMab-induced cell proliferation was assessed after 3 days incubation of whole-blood samples, diluted 1:10 in RPMI 1640 containing different mixtures of BsMab, IL-2, and irradiated target cells, as indicated, for 72 h at 37°C, 5% CO2. Per 100-µl cell sample, 1 µCi [3H]thymidine (Amersham, Chalfont, United Kingdom) was added to each well and incubated for an additional 16 h at 37°C, 5% CO2. All determinations were done in triplicate. After the incubation, cells were harvested onto glass fiber filters. Per filter, 1 ml Hisafe3 scintillation fluid (LKB, Uppsala, Sweden) was added, and the filters were counted by scintillation counting.

**Cytotoxicity.** Cytolytic activity was assessed using a standard 51Cr-release assay. GLC-1M13 target cells were labeled with 100 µCi Na2CrO4 (Amersham) for 1 h at 37°C, 5% CO2, in 100 µl culture medium. After the incubation, cells were washed three times with culture medium and resuspended at a final concentration of 5 × 10^5/ml. From this cell suspension, 50 µl (2.5 × 10^3 cells) were added to each well of a 96-well microtiter plate in which 5 × 10^5 isolated, monocyte-depleted PBMC in 100 µl were present that had been preactivated in the microtiter plate for 24 h at 37°C, 5% CO2, with different BsMab in the presence of relevant (GLC-1M13) or irrelevant (GLC-1) irradiated target cells (see above). Fifty µl BIS-1 were added to give a final BIS-1 concentration of 0.1 µg/ml in a final volume of 200 µl. Alternatively, CTL cultured in bulk suspension in the presence of the mitogenic anti-CD3 antibody WT-32 and IL-2, as described above, were used as effector cells. Just prior to the cytotoxicity assay, effector cells were washed once with culture medium and suspended to a final concentration of 1 × 10^6/ml. Fifty µl of this cell suspension (5 × 10^5 cells) were added to each well of a 96-well microtiter plate and incubated with 2.5 × 10^3 target cells in the presence or not of different BsMab (0.1 µg/ml final concentration) and IL-2 (final concentration, 100 IU/ml). All determinations were assessed after a 4-h incubation in a total volume of 200 µl at 37°C, 5% CO2 in triplicate. The microtiter plates were centrifuged for 5 min at 1000 rpm, and 100 µl cell-free supernatant samples...
BISPECIFIC ANTIBODY-MEDIATED COSTIMULATION

Fig. 1. Cytolytic activity of CTL, preactivated using a combination of anti-CD3 mAb WT32 in combination with IL-2 (•) or freshly isolated resting PBMC (D), against GLC-1M13 target cells using an E:T ratio of 20. The potential of various BsMab to induce targeted T-cell cytotoxicity is depicted (data are the means of triplicate cultures; bars, SD). Differences between the test groups with or without IL-2 are all significant (P < 0.05). BIS-I, CD3 X EGP-2; BIS-18, CD5 X EGP-2; BIS-20, CD28 X EGP-2.

were collected and counted for 5 min in a LKBG gamma counter (LKB). Cell lysis was calculated according to the formula:

% cell lysis = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Maximal release} - \text{spontaneous release}} \times 100.

Maximal release was determined from a sample to which Triton X-100 solution was added to a final concentration of 1% (v/v) instead of BsMab, IL-2, and effector cells. Spontaneous release was determined from a sample to which medium was added instead of effector cells.

Statistical Analysis. Unless stated otherwise in the figure legend, each experiment was performed at least three times in triplicate. The mean ± SD of a representative experiment is depicted in the figures. Statistical significance of the different test groups was analyzed using the Wilcoxon test for unpaired observations in which P < 0.05 was considered significant.

RESULTS

T-Cell Activation Is a Prerequisite for Effective BsMab-mediated Cellular Cytotoxicity. To test the cytotoxicity-directing potential of the various BsMab, CD3/IL-2-activated CTL were incubated with various mixtures of the BsMab BIS-1 (anti-CD3/anti-EGP-2), BIS-18 (anti-CD5/anti-EGP-2), and BIS-20 (anti-CD28/anti-EGP-2). It appeared that activated CTL show high cytolytic capacity towards EGP-2-positive GLC-1M13 target cells when incubated with any BIS-1-containing BsMab mixture (Fig. 1), whereas freshly isolated resting PBMC displayed only very limited cytolytic activity. The EGP-2-negative target cell line GLC-1 was not lysed by activated CTL or freshly isolated PBMC, irrespective of the BsMab used (data not shown). Apparently, the cytolytic potential of T cells that had been activated by a protocol consisting of a combination of CD3 signaling and IL-2 could not be enhanced by the addition of costimulatory signals, such as provided by BIS-18 and/or BIS-20, i.e., CD5 and/or CD28.

BsMab-mediated Activation of T Cells by Cross-Linking via CD3, CD5, and CD28. Next, the potential of various BsMab to induce stimulation of resting T cells via interaction with EGP-2-positive target cells was investigated. BIS-1 was used in these experiments to involve the CD3 signaling pathway, whereas BIS-18 and BIS-20 were used to provide costimulatory signals via CD5 and CD28, respectively. T-cell activation was monitored by analysis of the expression of the early activation marker CD69 and by assessing the proliferative and cytolytic activity of the various cultures. The experiments were done in whole-blood cultures, which better mimic the in vivo situation, and to avoid any monocyte activation due to PBMC isolation procedures (30). When IL-2, EGP-2-positive target cells and anti-EGP-2-directed BsMab mixtures were present, the CD69 expression on both CD4 and CD8 T cells became up-regulated, especially when CD3 activation (i.e., via BIS-1) was involved (Figs. 2 and 3).
Bispecific antibody-mediated costimulation

Fig. 3. Flow cytometric analysis of CD69 expression by CD8-positive (A and B) and CD4-positive (C and D) lymphocytes after incubation of whole-blood samples with GLC-1M13 cells and BeMab as indicated for 24 h at 37°C, 5% CO2. The 24-h incubation was done in the absence (A and C) and presence (B and D) of 100 IU/ml recombinant IL-2. The number of cells detected (events) and the CD69 expression are shown in arbitrary units.

Costimulation by simultaneous ligation of CD3/CD5 (i.e., via BIS-1 and BIS-18), CD3/CD28 (i.e., via BIS-1 and BIS-20), or CD3/CD5/CD28 (i.e., via BIS-1, BIS-18, and BIS-20) induced an increased CD69 expression. T-cell stimulation by simultaneous ligation of CD3/CD28 in the absence of CD3 triggering resulted only in an increase in CD69 expression, which was comparable to activation via CD3 only. Activation via CD5 or CD28 only did not induce CD69 expression (data not shown). The effect of the costimulatory signals as provided by BIS-18 and BIS-20, on the expression of CD69 by peripheral blood T lymphocytes, was more pronounced in the absence of IL-2. Stimulation with BIS-1 alone resulted in only a marginal increase of CD69 expression on T cells, whereas costimulation via BIS-18 and BIS-20 induced an increment of CD69 expression (Fig. 2). Highest CD69 expression, both in CD4- and CD8-positive lymphocytes, was observed when both the CD3, CD5, and CD28 pathways were triggered simultaneously via BIS-1 together with BIS-18 and BIS-20 (Figs. 2 and 3). Activation via CD3 and CD5, or CD3 and CD28, could be enhanced by the addition of IL-2. Stimulation via the simultaneous triggering of CD3/CD5/CD28 resulted in a CD69 expression on T cells that could not be further enhanced with exogenously applied IL-2 (Figs. 2 and 3).

To further analyze the effects of BIS-1, BIS-18, and BIS-20 on the activation of T cells in the presence of EGP-2-positive target cells, the induction of effector cell proliferation and cytotoxicity was measured. In the presence of exogenously applied IL-2, enhanced proliferation was observed in whole-blood cultures when irradiated EGP-2-positive target cells were added together with BIS-1 but not BIS-18 or BIS-20 (Fig. 4). In the absence of exogenously applied IL-2, proliferation in response to BIS-1 alone was significantly lower (Fig. 4). More proliferation was induced by the simultaneous application of BIS-1 and

Fig. 4. Proliferation of whole-blood cell cultures in response to coincubation with irradiated GLC-1M13 tumor cells and various BeMab as indicated in the presence (●) or absence (□) of exogenous IL-2 (100 IU/ml). After 72 h, proliferation was assessed by [3H]thymidine incorporation. Values are depicted as the mean of triplicate cultures; bars, SD. *, significant difference compared to the same test group without exogenously added IL-2 (P < 0.05). BIS-1, CD3 × EGP-2; BIS-18, CD5 × EGP-2; BIS-20, CD28 × EGP-2.
BIS-18, or BIS-1 and BIS-20, whereas a comparable proliferation as seen with BIS-1 and IL-2 was seen after the simultaneous addition of BIS-1, BIS-18, and BIS-20 in the absence of exogenously applied IL-2. Moderate proliferation, which was somewhat enhanced by the addition of IL-2, was observed by the simultaneous addition of BIS-18 and BIS-20 in the absence of BIS-1. In the absence of tumor cells or the presence of an EGP-2-negative tumor cell line, no or only marginal induction of T-cell activation was observed, as was assessed by CD69 expression, proliferation (data not shown in the present report, see Ref. 36), and cytotoxicity (Fig. 6b).

In the presence of exogenously applied IL-2, costimulation with BIS-18 and BIS-20 was found to be differentially influenced by the concentration of BIS-1 (Fig. 5). Whereas a dose-dependent proliferation was found for BIS-1 when combined with BIS-18, this did not hold true for the combination of BIS-1 with BIS-20, which showed an optimum proliferation with low (0.01 μg/ml) concentrations of BIS-1. In the absence of exogenously applied IL-2, this phenomenon did not occur, and both costimulation via BIS-18 or BIS-20 showed a dose-dependent proliferation in response to increasing concentrations of BIS-1 (data not shown).

The same pattern as observed for CD69 expression and proliferation induction was found for the acquisition of cytolytic activity in response to stimulation with BIS-1, BIS-18, and BIS-20. In these assays, isolated PBMC depleted from monocytes were incubated with irradiated, unlabeled, EGP-2-positive (Fig. 6a) or -negative (Fig. 6b) target cells and various combinations of the different BsMab together with IL-2 or not. After 24 h, $^{51}$Cr-labeled, “fresh” GLC-1M13 target cells were added, together with BIS-1, to test the acquired cytotoxic potential. The induction of cytolytic potential was found to be dependent on the presence of EGP-2-positive GLC-1M13 cells during the preincubation period, although some background target cell lysis of 12% or less was induced by preincubation with EGP-2-negative GLC-1 cells in the presence of exogenously added IL-2 (Fig. 6b). In the absence of exogenously added IL-2, induction of cytolytic potential was less than 5% in all cases (data not shown). In the presence of exogenously added IL-2, the BsMab-mediated induction of cytolytic potential was comparable whether induction was done via a combination of CD3/CD5, CD3/CD28, or CD3/CD5/CD28 triggering (Fig. 6a). Thus, under the circumstance of CD3 triggering in the presence of exogenously added IL-2, neither CD5 nor CD28 ligation induced an increment of cytolytic potential. Interestingly, T-cell preactivation with the combination BIS-18 plus BIS-20, in the absence of BIS-1, also resulted in the induction of considerable cytolytic potential. This phenomenon proved to correlate with the induction of CD69 expression (Fig. 2) but less with the induction of proliferation (Fig. 4). In the absence of exogenously added IL-2, the possible role of CD5 and CD28 became apparent in the induction of a cytolytic potential of T cells. Without exogenously applied IL-2, activation via CD3 alone resulted in only a limited induction of cytolytic potential. Costimulation via CD5 or CD28 led to a significantly enhanced cytolytic activity. Activation via simultaneous ligation of CD3, CD5, and CD28 proved to induce a comparable cytolytic potential, as obtained with CD3 ligation in the presence of exogenously applied IL-2, whereas the cytolytic activity via CD3 together with CD5 and, to a lesser extent, CD3 together with CD28, could be further enhanced by the addition of IL-2 to the cytotoxicity assays (Fig. 6a).

**Discussion**

In this report, we investigated target cell-specific activation of T cells as induced by BsMab directed against the pancarcinoma-associated antigen EGP-2 and the T-cell surface receptors CD3, CD5, and CD28. To this end, the following BsMab were generated: BIS-1, directed against CD3 and EGP-2; BIS-18, directed against CD5; and EGP-2 and BIS-20, directed against CD28 and EGP-2. Activation of T cells was assessed by monitoring the induction of CD69 expression, which has been shown to be an early indication of activation because it is expressed at the cell surface of T cells as soon as 4 h after activation (18). In addition, the induction of proliferation and cytolytic capacity was measured. Expression of CD69 and cytolytic potential were assessed after effector-target cell interactions for 24 h, whereas proliferation induction proved to be only measurable after an interaction period of 72 h. Expression of CD69 and proliferation were assessed using whole-blood cultures (9, 10). In these cultures, monocytes are not preactivated, which normally occurs due to PBMC isolation procedures (30). Since the assessment of cytolytic activity by chromium-release assays is not feasible in whole-blood cultures, isolated monocyte-depleted peripheral blood lymphocytes were used for the detection of BsMab-mediated cytolytic activity.

In the presence of EGP-2-positive target cells, T-cell activation could be induced by using combinations of BsMab directed against EGP-2 on the one hand and CD3, CD5, or CD28 on the other. Triggering of T cells through combinations of BsMab directed against CD3/CD5 or CD3/CD28 could still be enhanced by exogenously applied IL-2, whereas T-cell activation that involved simultaneous CD3, CD5, and CD28 triggering could not be further enhanced by exogenously applied IL-2. T-cell activation by simultaneous ligation of CD5 and CD28 in the absence of triggering via CD3 has been described recently (37). Although we were not able to detect a clear proliferative T-cell response after simultaneous CD5/CD28 ligation without CD3 triggering, T-cell activation was apparent from an enhanced CD69 expression and an augmented acquired cytolytic potential, as measured in $^{51}$Cr-release assays.

* Unpublished observation.
Triggering of T cells via the TCR/CD3 complex only results in the initiation of activation, which is characterized by a rise in cytoplasmic calcium and protein kinase C translocation from cytosol to the membrane (38), but is also characterized by the production or activation of inhibitors that block IL-2 gene transcription and subsequently prevent proliferation (39). Furthermore, cytokine encoding mRNA is formed but seems highly labile and sensitive to degradation (40, 41). This most likely explains the fact that proper T-cell activation via CD3 is largely dependent on costimulatory signals, as provided by CD28 or the addition of exogenous IL-2. Although the exact signaling pathway via CD28 is as yet unclear, ligation of the CD28 molecule on T cells, together with CD3 cross-linking, results in an elevated proliferation (39). Furthermore, cytokine encoding mRNA is formed in the presence of exogenously applied IL-2, or not (3). After incubation for 24 h at an E:T ratio of 20, fresh 51Cr-labeled GLC-1M13 target cells were added together with 0.1 fig/ml BIS-1. Released 5'Cr was assessed after incubation for an additional 4 h. Values are depicted as the means of triplicate cultures; bars, SD. *, significant difference compared to the same test group without exogenously added IL-2. BIS-1, CD3 × EGP-2; BIS-18, CD28 × EGP-2.}

\[ \text{Fig. 6. Cytolytic activity of monocyte-depleted PBMC after preactivation with the indicated BsMab in the presence of irradiated GLC-1M13 (c) or GLC-1 (b). Preactivation was done in the presence (b) of exogenously applied IL-2 or not (c). After incubation for 24 h at an E:T ratio of 20, fresh 51Cr-labeled GLC-1M13 target cells were added together with 0.1 fig/ml BIS-1. Released 5'Cr was assessed after incubation for an additional 4 h. Values are depicted as the means of triplicate cultures; bars, SD. *, significant difference compared to the same test group without exogenously added IL-2. BIS-1, CD3 × EGP-2; BIS-18, CD28 × EGP-2.} \]
exogenously added IL-2, might be induced. The site-specific character of the T-cell activation might result in enhanced local effectiveness of the BsMab-targeted CTL, possibly also an enhanced tumor-specific homing and less systemic toxic side effects, as compared to BsMab treatments given in combination with exogenously applied IL-2.

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REFERENCES

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