Tumor Therapy with Bispecific Antibody: The Targeting and Triggering Steps Can Be Separated Employing a CD2-Based Strategy

Martin K. Wild, Wolfgang Strittmatter, Siegfried Matzku, Burkhart Schraven and Stefan C. Meuer

*J Immunol* 1999; 163:2064-2072; ;
http://www.jimmunol.org/content/163/4/2064

References
This article cites 45 articles, 21 of which you can access for free at:
http://www.jimmunol.org/content/163/4/2064.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Tumor Therapy with Bispecific Antibody: The Targeting and Triggering Steps Can Be Separated Employing a CD2-Based Strategy

Martin K. Wild, Wolfgang Strittmatter, Siegfried Matzku, Burkhart Schraven, and Stefan C. Meuer

For tumor therapy with unprimed effector cells, we developed a novel combination of a CD2 × tumor Ag bispecific targeting Ab and an anti-CD2 triggering Ab. These Ab constructs were derived from two novel CD2 mAbs, termed M1 and M2 that, together, but not individually activate T cells. Unlike many other CD2 Abs, M1 and M2 do not interfere with TCR/CD3 triggering nor do they inhibit binding of CD2 to its ligand CD8β, thus preserving the physiological functions of these important effector cell molecules. M2 was chemically conjugated with an Ab recognizing the epidermal growth factor-receptor (EGF-R). Incubation of unprimed peripheral blood mononuclear cells with the bispecific F(αb)2 construct (M2xEGF-R) in the presence of trigger Ab M1 led to efficient selective lysis of EGF-R-positive targets by CTL and NK cells. Importantly, the need for trigger Ab M1 for effector cell stimulation allowed to separate targeting from triggering steps in vitro and should thus enable to focus immune responses to sites of target Ag expression in vivo. The Journal of Immunology, 1999, 163: 2064–2072.

One concept to circumvent impaired effector-cell/tumor-cell interactions in malignant disease relates to the use of bispecific mAbs (BsAbs) that can simultaneously bind tumor cells and effector cells and activate the latter in a diseased organ. Several surface receptors expressed by immune effector cells have been suggested as targets for BsAbs such as Fc receptors on macrophages and neutrophils, the TCR/CD3 complex, and the CD2 and CD28 coreceptors (1). CD2 offers the advantage of being strongly expressed by all T lymphocytes and virtually all NK cells independent of their differentiation and activation stage (2). Potential effector cells expressing CD2 can be activated by pairs of monoclonal CD2 Abs, which leads to a vigorous proliferative response, massive cytokine production, and expression of cytolytic effector functions of both T lymphocytes and NK cells (3, 4).

In this paper we report on functional studies based on a novel mitogenic combination of CD2 mAbs, termed M1 and M2, respectively. Neither M1 nor M2 interact with the CD8β binding site of human CD2 and thus do not block CD2-mediated adhesion processes. In addition, both Abs are strongly reactive with resting T cells and, when used in combination, can trigger unprimed pre-effector cells to undergo differentiation into cytolytic effectors. M2 was chemically conjugated with an Ab fragment directed against the epidermal growth-factor receptor (EGF-R) yielding the bispecific Ab (BsAb) M2xEGF-R. If BsAb M2xEGF-R is applied together with M1, effector cells are induced to selectively kill EGF-R-positive tumor targets. Employing such a combination of BsAb plus triggering Ab, it should be possible to delineate a treatment strategy in which the targeting and activation steps can be separated. This could help to focus the cytolytic potential to sites of EGF-R expressing tumor targets and at the same time should avoid lymphocyte activation at unwanted sites and thus minimize systemic side effects of immunotherapy in vivo.

Materials and Methods

Cells

Human PBMC were prepared by Ficoll-Hypaque- (Pharmacia, Uppsala, Sweden) density centrifugation of heparinized whole blood. Resting human T cells were prepared as described in (5, 6) and were >95% reactive with CD3 mAbs. The following cell lines were used: human Jurkat T cell lymphoma line JM-P1 (7), human epidermoid carcinoma line A 431 (8), and human erythro-leukemia line K 562 (9). C8161 is a nonpigmented human melanoma cell line isolated from a recurrent malignant melanoma (10). This cell line was kindly provided by Dr. M. J. C. Hendrix (University of Arizona, Tucson, AZ). The cells were maintained in DMEM supplemented with 10% FCS and 2 mM l-glutamine. Single cell suspensions were derived after treatment with 0.5 mM EDTA. The T cell clone C3F2 (11) (1 × 10⁶ cells) was grown in the presence of 40 U/ml recombinant human IL-2 (Biotest, Dreieich, Germany), 1 × 10⁶ freshly prepared human PBMC (irradiated with 60 Gy), and 1.5 × 10⁴ EBV-transformed B-lymphoblastoid LAZ 509 cells (irradiated with 60 Gy). After 7–10 days, no live PBMC and LAZ 509 cells could be detected, and the T cell clone was used for cytotoxicity assays. Tumor infiltrating lymphocytes (TIL) have been prepared as described previously from freshly resected melanoma tissue (12) and have been stimulated via mixed lymphocyte tumor cell culture (MLTC) (13). In short, the freshly isolated lymphocytes were cocultured with autologous irradiated melanoma bulk culture tumor cells in RPMI 1640 medium supplemented with 20 U/ml IL-2 (Genzyme, Cambridge, MA) and 10% FCS. Restimulation of the bulk culture was done on day 12. MLTC derived bulk culture TILs have been used to perform cytotoxicity assays.

Monoclonal Abs

Abs M1 (= AICD2.M1, IgG1), M2 (= AICD2.M2, IgG1), and AICD2.5 (IgG1) were produced in our laboratory. In short: BALB/c mice were immunized with a sonicated lysate of S9 cells transfected with human CD2. Spleen cells isolated from immunized animals were fused with Ag8-PA1 (provided by M. Kramer, Institut für Immunologie, Heidelberg, Germany).
HAT/HT selection was done according to standard protocols. Screening of CD2 specific hybridoma supernatants was done by measuring Ab binding to Jurkat T cells in cytometry analyses. Hybridomas have been subcloned twice. Other CD2 mAbs, OKT11 (IgG1; Ortho, Neckargemünd, Germany), T112 (1OLD4C1, IgG2A), T113 (1 MONO 2 A6, IgG3; T112, and T113, were kindly provided by Dr. E. Reinherz, Dana-Farber Cancer Institute, Boston, MA), ICRFCD2.4 (IgM), ICRFCD2.5 (IgG2A), and ICRFCD2.8 (IgG2A; ICRF mAbs were kindly provided by Dr. M. Crumpton, Imperial Cancer Research Fund, London, U.K.). Further mAbs were CD3 mAb OKT3 (IgG1; Ortho), CD58 mAb AICD58.5 (IgG2A (14), and EGF-R mAb 425 (IgG2A (15)). Abs were used as protein A-purified murine Ig unless indicated otherwise.

**Preparation of BsAb and Ab fragments**

BsAb M2xEGF-R was generated by chemical recombination of Fab' fragments of CD2 mAb M2 and EGF-R mAb 425 as described by Brennan et al. (16). Briefly, Ab M2 and mAb 425 were converted into Fab'1 fragments by limited proteolysis with pepsin. Fab'2 fragments were purified by chromatography on protein A Sepharose. Fab' fragments were generated by a mild reduction with DTT (0.5 mM; Merck, Darmstadt, Germany). The CD2-specific Fab' fragments were modified with 5,5'-dithio-bis-2-nitro-benzoic acid (DTNB, Merck). Subsequently, bispecific F(ab')2 fragments were generated by conjugation of the Fab'-TNB (thio-bis-2-nitro-benzoic acid) derivative with the hinge-SH groups of the mAb 425-derived Fab' fragments. Purification of intermediate products (Fab', Fab'-TNB) as well as the final purification of BsAb was performed by gel filtration on a Superdex 200 column (Pharmacia). Purity of the bispecific conjugate was assessed by 10% nonreducing SDS-PAGE and by chromatography on a hydroxyapatite column. The Fab' fragments of the triggering Ab M1 were generated by limited proteolysis with pepsin. Fragments were purified as described.

**Sheep erythrocyte rosetting assays**

The CD2 molecule of human T cells binds to the CD58 molecule (T11TS). CD2-specific hybridoma supernatants was done by measuring Ab binding to Jurkat T cells in cytometry analyses. Hybridomas have been subcloned twice. Other CD2 mAbs, OKT11 (IgG1; Ortho, Neckargemünd, Germany), T112 (1OLD4C1, IgG2A), T113 (1 MONO 2 A6, IgG3; T112, and T113, were kindly provided by Dr. E. Reinherz, Dana-Farber Cancer Institute, Boston, MA), ICRFCD2.4 (IgM), ICRFCD2.5 (IgG2A), and ICRFCD2.8 (IgG2A; ICRF mAbs were kindly provided by Dr. M. Crumpton, Imperial Cancer Research Fund, London, U.K.). Further mAbs were CD3 mAb OKT3 (IgG1; Ortho), CD58 mAb AICD58.5 (IgG2A (14), and EGF-R mAb 425 (IgG2A (15)). Abs were used as protein A-purified murine Ig unless indicated otherwise.

**Cytotoxicity assays**

Cytotoxicity with T cell clones as effector cells was determined in standard 3Cr-release assays in round bottomed microtiter plates. Each well (tripli- cate cultures) received the respective Abs, effector cells, and 51Cr-labeled- target cells at the indicated concentration or number to give a total volume of 200 μl of supplemented RPMI medium (see proliferation assays). Six wells containing target cells in culture medium only were used for determining spontaneous 3Cr-release (SR). Six wells containing target cells in 1% Nonidet P-40 detergent were used for determining maximum release (MR). Cell mixtures were sedimented (230 × g, 5 min) and incubated at 37°C in a 6% CO2 atmosphere for 4 h. Finally, cells were pelleted (450 × g, 10 min), and 100 μl of supernatant was collected from each well to determine Chromium release in a gamma counter (LKB-Wallac, Stock- holm, Sweden). The percentage specific lysis was calculated as: 100 × [(experimental 3Cr release – SR)/(MR – SR)]. In experiments with PBMC as effector cells, these were incubated for 72 h with the respective Abs before 3Cr-labeled A 431 target cells were added and mixed with the effector cells. In some experiments unlabeled NK-sensitive K 562 cells were added additionally. Cell mixtures were sedimented (230 × g, 5 min) and submitted to a 4-h assay as described above. In assays with TILs, cells were incubated for the indicated times. Bispecific and monospecific Abs were added simultaneously or in two steps before supernatants were collected after a total culturing time of 18 h.

**Results**

**Anti-CD2 mAbs with a novel combination of characteristics**

For the design of CD2 × EGF-R-specific BsAbs we produced anti-CD2 mAbs with a combination of favorable characteristics. These mAbs (termed AICD2-M1 and AICD2-M2, abbreviated M1 and M2) are specific for human CD2 as assessed by binding to recombinant CD2 in a sandwich ELISA (data not shown). In competitive binding ELISAs, the mAbs M1 and M2 cross-blocked with the established anti-CD2 mAb T113 (3) in binding to recombinant CD2 (Fig. 1B). However, mAbs M1 and M2 differ from mAb T113 in that they strongly bind to CD2 on resting human T cells (Fig. 1A), whereas the T113 mAb binds to its CD2 epitope only upon T cell activation (3).

A well-recognized physiological function of the CD2 glycoprotein is to mediate cell-cell adhesion by binding to its ligand CD58 (19, 20). Competitive binding of the majority of CD2 mAbs, including mAb OKT11, blocks the interaction between CD2 and CD58 (Fig. 1 and Ref. 21). In contrast, the CD2 mAbs M1 and M2 neither when applied individually nor in combination exhibit such interference as judged from sheep erythrocyte rosetting assays (Fig. 1C). In this respect they resemble the CD2R Ab T113 (3).

Fig. 2A shows that the combination of mAbs M1 and M2 induces proliferation of PBMC as well as of purified T cells, whereas, individually, the Abs have no mitogenic effect. In the presence of suboptimal concentrations of mAbs M1 and M2, ad- dition of recombinant CD58 strongly enhances this proliferative response (Fig. 2B). CD58 specificity of costimulation was proven by the addition of an anti-CD58 mAb, which abrogated the func- tional effect of recombinant CD58.

Fig. 2C shows that several of the anti-CD2 mAbs tested, including mAbs T112 and T113, inhibit the in vitro activation of PBMC via the TCR/CD3 complex. mAb OKT11 was not included in this assay but had previously been reported to inhibit CD3-driven pro- liferation of T cells as well (21). In contrast, the mAbs M1 and M2 do not interfere with the TCR/CD3-driven proliferation of PBMC (Fig. 2C).

**Production of BsAb M2xEGF-R**

The CD2 specific mAb M2 as well as EGF-R mAb 425 were cleaved into Fab'1 fragments and converted into Fab' fragments. Conjugation of Fab' fragments of mAb 425 and TNB-derivatives of M2

**Production of BsAb M2xEGF-R**

The CD2 specific mAb M2 as well as EGF-R mAb 425 were cleaved into Fab'1 fragments and converted into Fab' fragments. Conjugation of Fab' fragments of mAb 425 and TNB-derivatives of M2

**Flow cytometry and analysis of CD2 modulation**

Flow cytometry studies of Ab recognition by mono- and bispecific Abs were conducted as described elsewhere (18). Studies of CD2 modulation were performed as follows. Each well of a flat-bottom 24-well plate re- ceived 1 × 102 freshly prepared PBMC and the respective Abs in the concentration as indicated. Cultures with BsAbs were additionally mixed with 2 × 103 A 431 cells. All cultures had a final volume of 2 ml. Cultures were incubated for 18 h at 37°C in a 6% CO2 atmosphere to allow mod- ulation before they were washed two times and incubated for 30 min at 4°C with biotinylated CD2 mAb ICRFCD2.8 (10 μg/ml) for detection of sur- face-CD2. The negative control sample was incubated with culture medium instead. Following two washes cells were stained for 15 min at 4°C with steptavidin-FITC conjugate (Becton Dickinson, San Jose, CA), washed again, and analyzed on an EPICS Profile flow cytometer (Coulter Elec- tronics, Hialeah, FL).
Fab' fragments resulted in CD2 × EGF-R bispecific F(ab')2 fragments (BsAb M2xEFG-R). A yield of purified BsAb of 20–30% based on the F(ab')2 starting material was achieved and is demonstrated in Fig. 3. High purity of the bispecific conjugate was demonstrated by chromatography on hydroxylapatite (data not shown) as well as in nonreducing SDS-PAGE (Fig. 3).

**Binding properties and CD2-modulation by BsAb M2xEFG-R**

Flow cytometry studies showed that BsAb M2xEFG-R bound to both the CD2-positive T cell line JM-P1 as well as to the EGF-R-positive epidermoid carcinoma line A 431 (Fig. 4). As expected from the monovalent CD2 binding of the bispecific conjugate, JM-P1 staining by BsAb M2xEFG-R was weaker as compared with staining by the parental CD2 mAb M2 (Fab')2. When compared with the parental EGF-R mAb 425 (Fab')2, however, staining of A 431 cells was only moderately reduced.

Engagement of the CD2 glycoprotein by CD2 specific Abs can lead to its modulation. This is shown here for the incubation of PBMC with an anti-CD2 mAb of the IgM isotype (compare modulated cells in Fig. 5C with unmodulated cells in Fig. 5B). Modulation could also be expected in a “targeting” situation in which effector and target cells bind to each other via BsAbs and oligomerize CD2. A strong modulation of the CD2 receptor would not allow repeated binding of effector cells to targets nor would a second challenge with BsAb be able to target and activate such effector cells. Therefore, we tested whether the CD2 molecule remains detectable on the surface of effector cells following incubation with BsAb M2xEFG-R and M1 F(ab')2 in the presence of A431 target cells. Fig. 5D shows that modulation of CD2 in PBMC effector cells during a 16-h incubation in vitro was moderate. The CD2 molecules remained detectable with a reduction of the available surface CD2 of about 41%.

**BsAb M2xEFG-R mediates EGF-R-specific target cell lysis by a cytotoxic T cell clone**

To induce lysis of tumor cells, BsAb M2xEFG-R has to mediate binding of effector cells to target cells (targeting) and to activate effector cells to trigger their cytolytic function. To investigate the targeting properties of BsAbs, cytotoxicity assays were conducted employing a cytotoxic CD8 T cell clone (C3F2) (11). Before assays, C3F2 cells were cultivated in the presence of IL-2 and feeder cells (irradiated PBMC plus EBV-transformed B cells). Since in such a differentiation state further activation is not required for cytolytic action, incubation with BsAb alone should be sufficient to achieve tumor cell lysis. Fig. 6 shows that, whereas the parental mAbs M2 (Fab')2 and 425 (Fab')2 were unable to mediate A431 cell lysis, BsAb M2xEFG-R induced strong cytotoxicity indicating efficient targeting to the effector cells. Furthermore, lysis of A 431 cells was due to the cytolytic function of the effector cells and not to a direct toxic effect of BsAb M2xEFG-R on the targets since the
incubation of A431 cells with the BsAb in the absence of effector cells did not induce lysis (data not shown). Specificity of BsAb-mediated targeting was tested by adding an excess of EGF-R mAb 425 (Fab')2 to cultures of C3F2 T cells, target cells and BsAb. Fig. 6 shows that the addition of EGF-R mAb led to an almost total inhibition of A 431 cell lysis thus proving EGF-R specificity. Furthermore, the inserted diagram in Fig. 6 demonstrates that BsAb M2xEGF-R, even at a high concentration of 250 ng/ml, did not induce lysis of the EGF-R-negative target K562 by C3F2.

BsAb M2xEGF-R plus trigger Ab M1 activate resting PBMC for target cell lysis

In a further set of experiments, we investigated whether the combination of BsAb M2xEGF-R and M1(Fab')2 would be able to activate resting PBMC to undergo proliferation. As shown in Fig. 7, this is indeed the case. When added individually, however, these reagents exerted no stimulatory effect.

Activation of PBMC by BsAb M2xEGF-R plus trigger Ab M1 (Fab')2, as observed in proliferation assays also induced the cytolytic effector function in resting T cells: Fig. 8 shows that this combination of Abs was capable of activating PBMC to lyse EGF-R-positive A 431 cells. Again individual Abs were not able to mediate tumor cell lysis. To test the BsAb-mediated contribution to cytolyis by CD2+ NK cells, which represent a subset of PBMC, unlabeled NK target cells (K 562) were added to the mixed cultures of PBMC and labeled A 431 cells (cold target inhibition). Fig. 8 shows that the addition of K562 cells approximately halved the specific lysis of A 431 cells indicating a considerable contribution of NK cells to the BsAb-mediated cytotoxic activity of PBMC.
BsAb M2xEGF-R plus trigger Ab M1 mediate cytotoxicity in a two-step protocol

In a final set of experiments, we investigated whether it is possible to target effector cells to tumor cells in a first step by giving BsAb alone and to trigger the former in a second step, i.e., 12 h later by M1 (Fab')2 to kill. We made use of a human allogeneic system employing the EGF-R-positive C8161 melanoma cell line as target along with MLTC-derived freshly prepared tumor infiltrating lymphocytes. Fig. 9A shows that dose dependently the combination of BsAb M2xEGF-R plus M1 (Fab')2, when added simultaneously, induced tumor cell lysis while the individual reagents did not. The efficiency of this TIL driven melanoma specific lysis was similar as for the PBMC/A431 system situation (compare Fig. 8). Importantly, virtually analogous results were obtained when the addition of BsAb M2xEGF-R and triggering-mAb M1 F(ab')2 was separated by 12 h (Fig. 9B). This result opens up the possibility to employ the CD2-based approach delineated here to separate targeting from triggering steps in a potential therapeutic setting.

Discussion

Novel anti-CD2 mAbs were produced by selection for mitogenicity for T cells as well as for noninterference with additional intrinsic T cell functions. The combination of mAbs M1 and M2 was

FIGURE 5. BsAb M2xEGF-R causes limited modulation of the CD2 molecule. Freshly prepared PBMC were incubated for 16 h at 37°C with culture medium (A and B), CD2 mAb ICRFCD2.4 (IgM, 1:400 dilution of ascites) (C), BsAb M2xEGF-R (50 μg/ml), mAb M1 F(ab')2 (50 μg/ml), and A 431 cells (D). The PBMC/A 431 cell ratio in D was 5:1. Cultures (except A) were then incubated with biotinylated CD2 mAb ICRFCD2.8 (10 μg/ml), which could not be blocked by the Abs used in the previous incubation. Negative control (A) was incubated with culture medium instead. Cells were stained with streptavidin-FITC conjugate and analyzed on a flow cytometer. Numbers without brackets represent mean fluorescence intensities in arbitrary units. Numbers in brackets show the percentages of positively stained cells.

FIGURE 6. BsAb M2xEGF-R mediates EGF-R-specific lysis of target cells by the cytotoxic T cell clone C3F2. 3HCr-labeled A 431 target cells (2 × 10⁴) were incubated with 2 × 10⁴ C3F2 T cells and the following Abs: BsAb M2xEGF-R (‚), CD2 mAb M2 F(ab')2 („), EGF-R mAb 425 F(ab')2 („), and BsAb M2xEGF-R plus mAb 425 F(ab')2 (50 μg/ml) (ı). Inset, C3F2 effector cells and targets (A 431 and K 562 cells) were cultured in a ratio of 3:1 without Ab (open columns) or in the presence of an optimal concentration of the BsAb M2xEGF-R (250 ng/ml) (filled columns, compare dose-response curve (ı)). Data are expressed as percentage of specific lysis in a 4-h assay and represent means of triplicate cultures ± SD.

FIGURE 7. BsAb M2xEGF-R induces proliferation of PBMC when complemented by trigger Ab M1 F(ab')2. PBMC (5 × 10⁴) were cultured for 72 h in the presence of M2xEGF-R + M1 F(ab')2 (●), M2xEGF-R (△), or M1 F(ab')2 (◇). Cells were pulsed with 37 kBq [3H]thymidine and harvested following 16 h of additional incubation. Values represent means of triplicate cultures ± SD.
selected and proved to be highly mitogenic for human T cells and PBMC. The requirement of two different CD2 mAbs to activate human T cells is a common characteristic of mitogenic CD2 mAbs (22) and represents the basis for a novel concept of BsAb-mediated tumor therapy in which the targeting and triggering steps can be separated.

MAbs M1 and M2 exhibited cross-blocking with the previously described comitogenic CD2 mAb T113 in binding to the CD2 molecule. However, mAbs M1 and M2 recognize the CD2 molecule on resting T cells, in contrast to T113 (3), suggesting that the novel mAbs and mAb T113 bind to overlapping but not identical epitopes (23). Recognition of CD2 on resting T cells is a prerequisite for their use as parental mAbs to form bispecific conjugates that are designed to target unprimed effector cells to particular sites in vivo.

The novel CD2 mAbs were tested for noninterference with the CD2-CD58 interaction. The rationale behind this was that bispecific Abs used in, e.g., cancer treatment should not block important T cell functions involving the CD2-CD58 interaction, such as cell-cell adhesion, recirculation of T-lymphocytes as well as costimulation in the activation of T cells through CD2 (17, 19, 24). Unlike mAb OKT11 (which had been used as parental CD2 mAb for a trispecific CD2 × CD3 × TA construct) (25) and several other CD2 specific Abs, mAbs M1 and M2 did not interfere with the CD2-CD58 interaction. Consequently, recombinant CD58 proved to be able to exert its costimulatory function in the presence of parental CD2 mAbs M1 and M2. Thus, in cancer therapy, two effects can be expected: T cells that have bound therapeutic Abs of M1 or M2 specificity but have not yet found access to tumor cells would still possess their CD2-mediated adhesion functions. Secondly, T cells which have bound to tumor cells via a CD2 × TA bispecific conjugate may receive an additional stimulus from binding of CD58 expressed on the tumor cells that would augment their cytotoxic activity.

In this regard, unlike several other costimulatory ligands (CD80, CD86, CD54), CD58 is expressed in the majority of tumor cells of various derivations investigated so far (26) and therefore could enhance the Ab-driven activation of different types of effector cells.

CD2 mAb OKT11 when applied in unconjugated form blocks T cell activation via the TCR/CD3 complex (21) through an as yet unknown mechanism whereas as mentioned above (25) in the form of a T113 × CD3 × target cell trispecific Ab strongly enhances lysis. We have examined both parental CD2 Abs M1 and M2 and other CD2 mAbs for interference with this kind of activation. In contrast to mAbs T112, T113, and a series of additional CD2 mAbs, M1 and M2 did not reduce TCR/CD3-driven proliferation and were even slightly comitogenic. As long as the precise molecular mechanisms underlying lymphocyte triggering by mAbs are not elucidated, explanations regarding receptor clustering processes or intracellular events that might be responsible for these
phenomena will remain pure speculations. However, at least one point can be made: there is ample experience from studies on large Ab panels, e.g., CD clusters that have clearly demonstrated that functional Ab activities often relate to molecular features of the Abs themselves (which in spite of extensive investigations on their avidities, isotopes, or molecules/epitopes to which they bind are far from being understood). In this regard it should be mentioned that one of our criteria for the selection of M1 and M2 was that they should not interfere with the CD3 initiated mode of activation in vitro so that negative side effects such as immunosuppression during in vivo application of M1- and M2-derived (Fab')2 fragments and BsAbs would become rather unlikely. Perhaps more importantly, when compared with the widely used targeting to the CD3 complex, the M1- and M2-based targeting of the CD2 molecule may have a critical advantage: previous in vitro studies have shown that binding of CD3 × TA BsAbs to CTL can lead to the inability of these cells to carry out a TCR-specific target cell lysis (27). This phenomenon may be due to inhibition of activation via the TCR/CD3 complex. Alternatively, a CD3 × TA BsAb-mediated reduction of surface expression of the TCR/CD3 complex (modulation) (28) is possible, which would lead to an inability to bind MHC/Ag complexes. Moreover, exposure of T cells to CD3 mAb alone can induce anergy (29). Indeed, we have observed that a CD3 × EGF-R BsAb leads to a strong loss of cell surface expression of the TCR/CD3 complex (our unpublished data). These unwanted reactions can be excluded for BsAbs with M1- or M2-specificity because these mAbs neither inhibit activation via TCR/CD3 (Fig. 2C) nor do they modulate the TCR/CD3 complex (data not shown).

With regard to the Ag on target cells, the EGF-R was chosen. The EGF-R has been reported to be highly over-expressed on malignant cells like epidermal and colorectal carcinoma cells (30) besides its normal expression on endothelial cells, fibroblasts, glial cells, and other cell types. Its high over-expression on certain tumors should allow to find a range of Ab concentrations that cause preferential damage to malignant cells. Moreover, unlike many Ags that are more specific for malignant cells, the EGF-R does not bear the disadvantage of a strong heterogeneity of expression within a given tumor (31).

Binding of particular Abs to triggering receptors can result in their total loss from the cell surface through modulation (32–34). In our experiments, coincubation of PBMC with M2 × EGF-R, trigger Ab M1(Fab')2, and A 431 target cells (imitating the potential targeting situation in vivo) led to a 41% reduction of the cell surface density of CD2. Maintained CD2 expression may enable T cells to bind further BsAbs if a therapy with repeated administration of BsAb (35) is chosen.

Cytotoxicity assays with a CD8+ T cell clone showed that BsAb M2xEGF-R was able to target T cells to EGF-R-positive A 431 tumor cells with the bispecific conjugate being active at nanogram quantities. Inhibition of target cell lysis by the addition of EGF-R mAb as well as unreactivity toward an EGF-R negative cell proved the specificity of M2xEGF-R -mediated targeting.

The performance of M2xEGF-R in the activation of unprimed effector cells was tested in both proliferation and cytotoxicity assays. In these assays the BsAb has to be supplemented by the trigger Ab M1(Fab')2 to achieve activation. Proliferation assays showed that the M2 moiety in BsAb M2xEGF-R maintained its ability to stimulate lymphocytes in concert with M1(Fab')2.

Cytotoxicity assays showed that stimulation by M2xEGF-R and M1(Fab')2 induced cytotoxic activity in human PBMC against EGF-R-positive target cells at Ab concentrations similar to those inducing proliferation. No previous study analyzing the performance of a combination of CD2 × TA BsAb and CD2-trigger-Ab (36–38) had demonstrated that such a combination of Abs can induce cytotoxicity in resting PBMC. Given that the majority of NK cells express functional CD2 molecules, it was not unexpected that the cytolytic activity of PBMC was to ~50% due to NK activity as judged from cold target inhibition assays using the standard NK target K562.

The ability of the Ab combination M2xEGF-R plus M1(Fab')2 to activate and recruit NK cells for tumor cell neutralization should be considered an important advantage over the function of BsAbs with CD3 specificity. The simultaneous recruitment of T and NK cells as indicated by our in vitro experiments may provide the basis of an enhanced NK activity because T lymphocytes can strongly support NK functions by secreting IL-2.

Further differences between CD2- and CD3-directed BsAbs exist: In CD3 × TA F(ab')2, BsAbs the monovalency of the anti-CD3 moiety may cause problems because effective CD3-mediated T cell activation is dependent on cross-linking of CD3 molecules (39). In accordance with this fact, triggering of T cells with monovalent reagents requires cross-linking of CD3 molecules by the simultaneous binding of BsAbs to CD3 on T cells and Ags on tumor cells (40). Often tumor cells have reduced surface densities of tumor Ags due to immunoselection (41) or modulation (42). This may cause insufficient cross-linking of the CD3 molecules on T cells if BsAbs are chosen that are specific for such tumor Ags. Activation of T cells via CD2, however, can be achieved by the use of soluble monoclonal CD2 mAbs (Ref. 3 and Fig. 1A) or BsAb M2xEGF-R plus mAb M1(Fab')2, without cross-linking (Fig. 7). Thus, here, cross-linking cannot become a limiting factor for T cell activation.

The “two signal hypothesis” of T cell activation (43) demands a second signal delivered by binding of ligands to accessory TCRs in addition to the first signal delivered via the TCR/CD3 complex. However, ligands which normally deliver such second signals, like the molecules of the B7 family, are not expressed on many solid tumors (44). Thus, second signals may not be sufficiently available in the case of tumors that would limit the application of CD3 × TA BsAbs. In such cases the combination of a CD2 × TA BsAb and trigger CD2 mAb may be more efficient because binding of two different CD2 Abs provides both signals required for T cell activation (45). Moreover the majority of malignant cells investigated in our laboratory express at least some CD58 molecules which amplify the CD2-dependent mode of T cell and NK cell activation (26).

The requirement of two Abs for activation of T cells via CD2 opens the perspective of a two-step concept of tumor therapy. Lymphocytes may be loaded ex vivo with BsAb M2xEGF-R before their injection into the blood stream. Alternatively BsAb M2xEGF-R could be administered i.v. Ab-coated lymphocytes or BsAb M2xEGF-R should then be targeted to tumor sites. Importantly, effector cells are not activated during this “targeting phase” because they lack binding of the second CD2 Ab. The length of the targeting phase can be optimized by preceding biodistribution analyses (currently performed in tumor xenotransplanted mice) in which the time of maximal tumor infiltration by BsAb-armed lymphocytes can be determined. In this work a time range of up to 12 h has been tested in vitro (Fig. 9). The 12-h time range is far off the overall half-life of murine F(ab')2 Ab fragments in blood which is 6–8 h in experimental animals (46, 47). Thus, most of the circulating BsAb is expected to be either localized or cleared by the time the trigger Ab is given. In the second step (“activation phase”) trigger Ab M1(Fab')2 will be delivered which should activate only those lymphocytes that have been targeted by BsAb M2xEGF-R, thus allowing a preferential local activation. In this concept, BsAb-mediated side effects should be reduced by injecting the trigger Ab...
following a targeting phase of optimal length. Moreover, in contrast to a previously proposed strategy using a bispecific CD2 × TA Ab with a T11α moiety (36), the present concept based on M1 and M2 does not need ex vivo activation of CD2-positive lymphocytes before reination.

For the clinical application of CD2 × TA BsAbs a study by Riethmüller et al. (48) may be considered in which a mAb was used to target disseminated tumor cells in patients with colorectal cancer who had undergone curative surgery. The easy access to unshielded disseminated tumor cells for Abs as well as for effector cells may represent a favorable situation for the application of CD2 × TA BsAbs like M2xEGF-R especially when considering the biodistribution pattern of NK cells. In solid tumors these cells are found in rather small numbers (49), whereas disseminated cells may be ideal targets for BsAb-redirected NK cells. Finally, besides their therapeutic application in vivo M1 plus M2xEGF-R Abs could be valuable for ex vivo tumor cell purging.

To prepare future in vivo studies, Abs M2xEGF-R and M1(Fab)2 have been successfully tested for their in vitro performance in two-step cytotoxicity assays (Fig. 9) and for their in vitro function in autologous systems (W. Strittmatter, C. Jäggle, and S. Matzku, unpublished data). Furthermore, the kinetics of biodistribution of M2xEGF-R have been determined in experimental animals to gain data on the optimal period of time needed for targeting by the BsAb (W. Strittmatter, C. Jäggle, and S. Matzku, manuscript in preparation). Mice transgenic for human CD2 (23, 50) exist and can be employed to determine the in vivo perfor-

Acknowledgments

We thank Drs. M. Crompton and E. Reinherz for donating mAbs. We also thank Dr. C. Jäggle for her help with the cytotoxicity assays and S. Nick, N. Bahl, D. Müller-Pompalla, and H. Kirchgessner for their excellent technical assistance.

References