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Simultaneous Activation of T Cells and Accessory Cells by a New Class of Intact Bispecific Antibody Results in Efficient Tumor Cell Killing

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Bispecific Abs (bsAb) are promising immunological tools for the elimination of tumor cells in minimal residual disease situations. In principle, they target an Ag on tumor cells and recruit one class of effector cell. Because immune reactions in vivo are more complex and are mediated by different classes of effector cell, we argue that conventional bsAb might not yield optimal immune responses at the tumor site. We therefore constructed a bsAb that combines the two potent effector subclasses mouse IgG2a and rat IgG2b. This bispecific molecule not only recruits T cells via its one binding arm, but simultaneously activates FcγRII accessory cells via its Fc region. We demonstrate here that the activation of both T lymphocytes and accessory cells leads to production of immunomodulating cytokines such as IL-1β, IL-2, IL-6, IL-12, and DC-CK1. Thus this new class of bsAb elicits excellent antitumor activity in vitro even without the addition of exogenous IL-2, and therefore represents a totally self-supporting system. The Journal of Immunology, 1999, 163: 1246–1252.

Successful immune responses against neoplastic cells in vivo depend on the cooperation of different classes of immune cells. Malignant cells may be targets for cytotoxic T cells that recognize specific MHC/peptide complexes on the cell surface or they may be eliminated by NK cells or monocytes/macrophages. However, these different classes of effector cell depend on each other with respect to the production of cytokines and the delivery of costimulatory signals and, therefore, they usually operate in a concerted manner. T cells are thought to be the most important subpopulation for killing of neoplastic cells. But full activation of naive T cells depends on proper Ag presentation by professional APCs or activated accessory cells and costimulatory molecules like CD40, LFA-3, B7.1, and B7.2 in the presence of cytokines such as IL-2 and IL-12 (1, 2). Tumor cells normally do not express B7 molecules, and instead of activating specific T cells, they may be able to simultaneously activate FcγRI accessory cells (via its Fc region) in the vicinity of tumor cells. In contrast to a similar T cell-redirecting bsAb, SHR-1, with the subclass combination mouse IgG2b × rat IgG2b (8), BiUII does not depend on the addition of exogenous IL-2 to provide full antitumor activity. This reveals the importance of the subclass combination for induction of activation signals via the Fc region of accessory cells. Moreover, we demonstrate that the antitumor efficiency of our bsAb is strongly reduced when T cells or accessory cells alone are used as effector cells. We therefore postulate that a “Tri-cell-complex” consisting of tumor cells, T lymphocytes, and accessory cells is created by this new class of bsAb. Only the formation of this complex results in a full activation of different effector cell types providing optimal antitumor efficiency.

Materials and Methods

Cell lines and PBMC preparation

Fadu (American Type Culture Collection, Manassas, VA) and PCI-1 are Ep-CAM-positive established squamous carcinoma cell lines of the head and neck (SCCHN) and were maintained in DMEM with 10% FCS. Both cell lines express epithelial cell adhesion molecule (Ep-CAM), epithelial cell adhesion molecule, and MHC class I, but not MHC class II as tested by flow cytometry (data not shown). DG75 is an EBV-negative Burkitt lymphoma cell line. PBMC were isolated from heparinized blood of voluntary donors by Ficoll density centrifugation. Where indicated (=PBL), the monocyte/macrophage fraction was removed by adhesion to plastic flasks twice for 2 h at 37°C in an incubator.

Monoclonal Abs

The following hybridomas have been used: 26II6 (rat IgG2b, anti-CD3; kindly provided by R. Schuh, Gesellschaft für Strahlung und Umweltforschung (GSF), Munich, Germany) and C215 (mouse IgG2a, anti-Ep-CAM; kindly provided by M. Dohlsten, Pharmacia Upjohn, Uppsala, Sweden).
RT-PCR
Total RNA from primary lymphocytes was isolated after up to 72 h of incubation with allogeneic SCCHN. The RNA preparations were treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. After inactivation of the enzyme, 1 μg RNA was reverse transcribed (Superscript Plus, Life Technologies, Gaithersburg, MD) with an oligo(dt) primer for 60 min at 42°C. PCR with one-tenth of the volume (2 μl) was performed in a buffer containing 1.5 mM MgCl₂, 100 pmol of each primer, 0.2 mM final concentration of each dNTP, and 0.5 μl Goldstar Taq polymerase (Eurogentec, Seraing, Belgium) in a final volume of 50 μl in an Perkin-Elmer (Norwalk, CT) thermal cycler. PCR primers are shown in Table I. Amplified bands were analyzed by electrophoresis in a 1.5% agarose gel and ethidium bromide staining.

Generation of dendritic cells (DC)
The adherent fraction of PBMC was incubated for 7 days in Iscove’s medium with 5% FCS (Life Technologies) and 800 U/ml of each human IL-4 and GM-CSF (both Boehringer Mannheim).

FACS analysis
For FACS analysis, 10⁵ cells were incubated with the primary Ab for 30 min on ice in PBS/5% FCS. The cells were washed twice in PBS and incubated for another 30 min with the second FITC-labeled Ab. After two final washings, propidiumiodide was added, and flow cytometry was performed using a FACSCalibur cytometer and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany). For isolation of highly purified CD²⁺ cells, PBMC were incubated with FITC-labeled Abs (PharMingen, Hamburg, Germany) and separated on a FACSCalibur.

Production of BiUII
The BiUII quadroma was produced as previously described (9). To isolate hybrid Ab molecules of the subclass combination rat IgG2b/mouse IgG2a from hybridoma supernatants, the supernatants were centrifuged, filtered, and loaded onto a 5 ml Econo Pac protein A column (Bio-Rad, Richmond, CA). After washing with 10 volumes of PBS, Ab with the hybrid heavy chain configuration was eluted with 0.1 M citric acid (pH 5.1).

Cell culture and killing efficiency
For determination of bsAb-mediated killing of tumor cells and cytokine production, 1 x 10⁵/well SCCHN (targets, =T) were pipetted in 96-well flat-bottom plates (Falcon, Franklin Lakes, NJ), and PBMC or subpopulations of these effector (=E) were added at ratios from 40:1 to 1:1 E:T. bsAb was used at 10 ng/well in a total volume of 100 μl/well RPMI with 10% FCS. Plates were incubated for 3 days at 37°C in a humidified atmosphere and 5% CO₂. All SCCHN were proven to express Ep-CAM and to lack costimulatory molecules CD80 and CD86.

MTT assay
To assess bsAb-mediated tumor cell killing, a colorimetric MTT-based assay was performed as described previously (10). Briefly, SCCHN target cells were plated in wells of 96-well flat-bottom plate and incubated overnight to prepare semiconfluent cell monolayers. Effector cells were added to the tumor cell monolayers at the appropriate ratios, and plates were incubated for 24–48 h. After removing effector cells by washing, MTT solution (0.5 mg/ml; Sigma, Deisenhofen, Germany) was added, and plates were incubated for a further 4 h. The MTT solution was removed, and blue crystals of formazan formed in viable tumor cells were dissolved by adding DMSO. Plates were read at 540 nm in a spectrophotometer, and the results were calculated based on the mean of absorbance obtained from at least six wells according to the following formula: % cell death = 100 x (C – E)/(C – B), where C is the optical density reading of the cells with target cells in the absence of effectors (control), B is background without any cell population, and E is the optical density reading of adherent tumor cells remaining in the wells after coincubation with effector cells.

Bioassays
Biologically active levels of IL-2, IL-6, TNF, and GM-CSF were measured in 96-well flat-bottom microtiter plates using cytokine dependent growth of cell lines CTLL-2, 7TD1, WEHI-164, and TF-1, respectively. Cell culture supernatants were titrated in duplicates and diluted from 1:5 to 1:10000. Standards of recombinant human IL-2 (PBH, Hannover, Germany), human IL-6 (Boehringer Mannheim), human TNF-α (PBH), or human GM-CSF (Boehringer Mannheim) were included in each assay to generate a standard curve. Intra- or interassay variability was less than 10% or 20%, respectively. Specificity of bioassays was confirmed by neutralizing active samples with cytokine specific Abs (PBH and Boehringer Mannheim). The lower detection limits of IL-2, IL-6, TNF, and GM-CSF were 20 pg/ml, 10 pg/ml, 0.1 ng/ml, and 10 pg/ml, respectively.

Results
Construction of BiUII
Ep-CAM is an Ag that is overexpressed on many carcinomas of different origin (11). Therefore, it was selected as a target tumor molecule for our bsAb. Because T cells are believed to be the most important effector cells for tumor cell elimination, they were targeted for activation via CD3. The bsAb constructed to recognize these two Ags was designated BiUII. Because most tumor cells do not express costimulatory molecules, BiUII was constructed with mAbs of subclasses that bind and activate human FcγR⁺ cells (12). The quadroma that resulted from the fusion of the anti-Ep-CAM and the anti-CD3 hybridomas was characterized and intact bsAb was purified as described (9). As shown in Fig. 1, BiUII represents a chimeric molecule consisting of the evolutionary related and highly homologous mouse IgG2a and rat IgG2b heavy chains.

Lysis capacity of BiUII
To determine the antitumor efficiency of BiUII, we first evaluated its capacity to mediate the killing of the Ep-CAM-positive tumor cell line PCI-1 (13) and compared it with the two monoclonal parental Abs (αCD3 and αEp-CAM). PCI-1 cells were cocultivated with PBMC in the presence of either BiUII, both parental Abs, or for control purposes, without Ab. After 2 days of culture, the numbers of remaining tumor cells were determined in a standard MTT assay. As shown in Fig. 2, BiUII displayed a much higher lytic capacity for tumor cells than equimolar concentrations of the corresponding parental Abs. Even 10-fold higher concentrations (50 ng/100 μl) of these Abs were less efficient than BiUII (data not shown). Theoretically, the two parental Abs also recruit FcγR⁺ cells and activate T cells via CD3. However, their observed antitumor capacity was much lower. This result suggested that BiUII-mediated formation of a complex involving at least two different classes of immune cells.
FcRα murine IgG2a chain (Fig. 1), it can theoretically bind and activate BiUII is a chimeric molecule consisting of a rat IgG2b chain and to ADCC by particular subclasses of opsonizing IgG Abs. Because Stimulation of T lymphocytes leads to IL-2 production and up-
stantiated the importance of accessory cells targeted via their Fc
PBMCs were used as the effector source of cells. This result sub-
shown in Fig. 4, maximal antitumor activity was achieved when whole PBMC for killing activity directed against PCI-1 cells. As
Because BiUII targets T lymphocytes and FcRα1 RI that is probably due to ADCC (16, 17). To determine whether BiUII could induce ADCC, we isolated macrophages and DC from peripheral blood by plastic adhesion and cocultured them with PCI-1 target cells with or without BiUII. The induction of ADCC measured in a MTT assay clearly demonstrated that PCI-1 cells were lysed much more efficiently in the presence of BiUII (Fig. 3). Because T lymphocytes were not present in this assay, as determined by FACS analysis (data not shown), the lysis of PCI-1 cells was most likely due to BiUII-mediated ADCC.
Accessory cells are necessary for optimal antitumor activity
Because BiUII targets T lymphocytes and FcγRI+ cells, both of which are activated by Ab binding, we questioned whether accessory cells contributed to the BiUII-mediated antitumor activity for example via direct phagocytosis. Therefore, we compared the killing capacity of unseparated PBMCs with highly purified T cell populations. CD2+ T cells were isolated by cell sorting and used in a MTT assay. We compared this purified T cell population with whole PBMC for killing activity directed against PCI-1 cells. As shown in Fig. 4, maximal antitumor activity was achieved when PBMCs were used as the effector source of cells. This result substantiated the importance of accessory cells targeted via their Fc receptors in tumor cell killing.
Induction of cytokine production
Stimulation of T lymphocytes leads to IL-2 production and up-
regulation of CD25, the a-chain of the IL-2R. IL-2 is the most
important autocrine growth factor for T cells (18). In addition, activated T cells induce IL-12 production by monocytes/macrophages and DC (19), thereby enhancing antitumor activity (20).
The high affinity FcγRI (CD64) is expressed by monocytes/ macrophages and dendritic cells (15). Because BiUII combines mouse IgG2a and rat IgG2b subclasses, it should theoretically be able to bind and activate FcγRI+ cells (12). Therefore, tumor cells opsonized by BiUII are complexed to accessory cells via CD64 and ADCC may be induced via direct phagocytosis. Subsequently, phagocytosed tumor-derived proteins can be processed and presented by MHC class I and II molecules, leading to humoral and cellular immunity. In addition, activated accessory cells can deliver cytokines like IL-6 and costimulatory signals via molecules like CD40, LFA-3, CD80, and CD86 that are mandatory for T cell activation and prevention of anergy (21).
Therefore, we determined whether BiUII was able to induce the production of IL-2 and IL-6 in PBMCs when cocultured in the presence of PCI-1 cells, thereby documenting the activation of both T cells and accessory cells. As shown in Fig. 5, PBMCs produce both cytokines only when BiUII is present. IL-2, which is mainly T cell derived, is produced in essentially equal amounts using either PBMCs or PBLs as effector cells. In contrast, IL-6 is

**FIGURE 2.** Lysis of PCI-1 cells. PBMCs from a healthy volunteer were incubated with PCI-1 in the presence of BiUII (5 ng/100 µl), both parental Abs (5 ng/100 µl each), or without Abs for 2 days. Regarding the lytic capacity, BiUII displays a much higher lytic activity compared with the parental Abs that were used simultaneously. One of five representative experiments is shown.

**FIGURE 3.** Killing of PCI-1 cells by monocytes and DC. Adherent cells derived from the peripheral blood of a voluntary donor were isolated by plastic adhesion and incubated for 2 days with PCI-1 with (50 ng/ml) or without bsAb. T cells were not detectable in the cell preparation. Killing of PCI-1 was determined in a MTT assay.

**FIGURE 4.** Only whole PBMC display full antitumor activity in the presence of BiUII (50 ng/ml). MTT assays with whole PBMCs and CD2+ T cells were performed, and the lysis of PCI-1 cells was determined. PBMC that contain FcγRI+ accessory cells show a much higher capacity for tumor cell killing than purified T cells, especially at lower E:T ratios. The results of one of three representative experiment are shown.
mainly produced by activated monocytes/macrophages and was only detectable when PBMCs were used but was not present when the adherent fraction was depleted. This finding supports our contention that the Fc region of BiUII mediates activation of FcγRI1 cells.

We also compared the bsAb with the two parental Abs for the production of IL-1β, IL-2, IL-4, IL-6, and IL-12. As depicted in Fig. 6, cytokine levels were only slightly elevated compared with background (IL-2 and IL-12), or were not induced at all (IL-1β, IL-4, and IL-6) when the parental Abs were used as stimulators. In contrast, after incubation with the bsAb, we observed a clear induction of all cytokines examined. These data and the fact that BiUII is much more efficient in tumor cell killing than the parental Abs suggest that the local production of various cytokines is likely a prerequisite for efficient tumor cell killing.

**BiUII-mediated production of IL-2 depends on the presence of the target molecule, Ep-CAM**

The major drawback in the in vivo application of bsAb is the problem of side effects provoked by uncontrolled cytokine release. In particular, the nonspecific activation of T cells via CD3 is thought to produce elevated IL-2 levels that can cause severe side effects. To evaluate IL-2 induction by BiUII, PBMCs were incubated with the Burkitt lymphoma cell line DG75 that does not express Ep-CAM, or with a subclone of DG75 that was stably transfected with an expression plasmid for Ep-CAM (DG75/Ep-CAM). As shown in Fig. 7, significant concentrations of IL-2 were only produced when PBMC were cultivated with DG75/Ep-CAM but not with DG75 cells. This finding implies that full activation of T cells and production of IL-2 is only achieved as part of a Tricell-complex involving accessory cells and tumor cells. A dependence on the presence of tumor cells expressing the target Ag Ep-CAM is an important parameter that can limit the risk of uncontrolled systemic IL-2 production by this bispecific reagent.

**Activation of DC**

DC are key regulators of immune responses. They form a system of efficient Ag presenting cells which present Ags to T cells (22) and trigger their activation via the CD40 dependent pathway (23). Recently, a DC-specific cytokine, DC-CK1, has been identified that is exclusively expressed by DC at high levels at sites of immune responses (24). DC-CK1 elicits a profound chemotactic activity to attract CD45RA+ naive T cells. Thus it is believed that DC are directly involved in the generation of cytolytic T lymphocytes (23, 25).

Because DC have recently been shown to express CD64 (15), we wanted to determine whether peripheral blood DC contribute to BiUII-mediated killing of tumor cells. The adherent fraction of PBMCs was incubated for 7 days in the presence of IL-4 and GM-CSF and then cocultured with PCI-1 cells with or without BiUII. Total RNA was then isolated at two different time points. Whereas after 4.5 h of incubation no substantial differences in DC-CK1 levels were detectable, the expression of the cytokine was clearly higher in the presence of BiUII 16 h later, indicating bsAb-mediated activation of DC (Fig. 8).

**Discussion**

We describe here the antitumor properties of a new class of bsAb. To our knowledge, this is the first investigation that demonstrates the simultaneous activation in vitro of different classes of immune effector cell by an intact bsAb. In addition to T lymphocytes that are recruited by one arm of BiUII, the Fc region binds and activates FcγRI+ cells, such as monocytes/macrophages and DC. This was demonstrated by the induced production of IL-1β, IL-2, IL-6,
IL-12, and DC-CK1 (Figs. 6 and 8). Obviously, this strong activation potential is correlated to the subclass combination mouse IgG2a × rat IgG2b that, in contrast to other reported combinations (e.g., mouse IgG2a × mouse IgG1 or rat IgG2b × mouse IgG1) (26), not only binds but also activates accessory cells.

Although T lymphocytes are believed to be the most important class of immune cell for the elimination of tumor cells, their activation depends on the presence of certain cytokines (most important IL-2) and so-called costimulatory molecules that are usually not delivered by tumor cells themselves. Rather, T cell activation depends on proper Ag presentation of tumor cell-derived peptides by professional APCs that have been demonstrated recently to be required for the induction of a long-lasting tumor immunity (27). New data corroborate the importance of costimulatory molecules for the prevention of activation-induced T cell anergy in immunotherapeutical trials (28). These are reasons that argue for our bispecific molecule, BiUII, that causes the simultaneous activation of both T cells and accessory cells.

Clinical trials with the 17-1A mAb demonstrated that CDC (complement dependent cytotoxicity) and ADCC most likely contribute to the observed antitumor effect. However, the most important class of effector cells, T lymphocytes, is not activated by this Ab (16). This may be a drawback because our in vitro assays with a comparable anti-Ep-CAM mAb revealed reduced activity in terms of tumor cell killing when compared with BiUII (Fig. 2). These results are consistent with investigations in different mouse tumor models demonstrating the benefit of the redirection principle by bsAb (Fig. 9) as compared with parental mAbs in vivo (29–32).

We also show by different methods that BiUII is much more efficient in inducing cytokine production than the two parental Abs (Fig. 6). Although the parental anti-Ep-CAM mAb is able to induce an antitumor ADCC (data not shown), an elevated cytokine production is not observed. The same holds true for the IL-2 production by T cells, which was only slightly increased after addition of the anti-CD3 mAb 26II6 in our experimental setting. Also, the BiUII-mediated production of IL-2 depends on the presence of the target Ag Ep-CAM. Therefore, our in vitro assays demonstrate that the interaction of T cells and accessory cells alone in the absence of Ep-CAM-positive target cells is not sufficient for the induction of IL-2 (Fig. 7), a fact that reduces the risk of intolerable systemic toxicity.
IL-2 concentrations during in vivo application of the bsAb. However, in strong contrast to other reported bsAb, BiUII activates T cells and accessory cells without the addition of exogenous IL-2, a fact that is particularly important for in vivo Ab application. In contrast, Klein et al. (8) demonstrated recently that exogenous IL-2 is necessary for a comparable activation of immune cells in vitro with the intact bsAb, SHR-1. Because SHR-1 is composed of the subclasses mlgG1 × rat IgG2b, these data argue for the necessity of two potent subclasses (like mlgG2a × rat IgG2b) to obtain optimal effector function leading to activation of accessory cells. Thus, the Tri-cell-complex represents a self-supporting system that produces cytokines that are necessary for efficient immune cell activation.

Thus, we postulate that the BiUII-mediated activation of accessory cells via its Fc region in addition to the recruitment of T cells contributes in two different ways to tumor cell destruction: 1) The contribution of accessory cells in the absence of T cells was demonstrated by cytotoxicity assays using the adherent fraction of PBMCs (Fig. 3). These data indicate that direct lytic killing mechanisms like apoptosis and phagocytosis or generally spoken, ADCD, exerted by different classes of accessory cells, improve tumor elimination. This holds true especially at decreasing E:T ratios as shown in Fig. 4, where a CD2 subset was compared with whole PBMC. 2) The activation of accessory cells is demonstrated by the production of cytokines like DC-CK1, IL-1β, IL-6, and IL-12. Our results provide evidence that the simultaneous activation of different effector cells clearly multipplies the antitumor efficiency (Figs. 2, 5, 6, and 8). Hence, we postulate that the formation of a Tri-cell-complex consisting of T cells, accessory cells, and tumor cells is required for optimal antitumor efficiency (Fig. 9), because a single class of effector cells or a combination of the two parental Abs (Fig. 2) was much less efficient.

bsAb are either used as intact molecules or as F(ab′)2 fragments. Whereas the antitumor activity is significantly higher with intact bsAb, F(ab′)2 fragments are considered to provoke less serious side effects in vivo (33). An excessive production of cytokines is often regarded as the major drawback and limiting factor for in vivo application of intact bsAb. However, in our mouse model system, a comparable bsAb (anti- MHC class II/anti-CD3) with the subclasses mIgG1 × rat IgG2b, these data argue for the necessity of two potent subclasses (like mIgG2a × rat IgG2b) to obtain optimal effector function leading to activation of accessory cells.

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