Chapter 2
Bispecific Antibodies from Hybrid Hybridoma

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2.1 Introduction

Hybrid hybridomas (also termed quadromas or tetradomas) are man-made cell lines that secrete bispecific antibodies (bsAb) with two different specificities being able to crosslink two distinct molecules. Such antibodies do not occur in nature and have been originally developed to improve immunohistochemical staining procedures and immunoassays (Milstein and Cuello 1983; Suresh et al. 1986). Interestingly, the fusion of two immunoglobulin-producing myeloma cells (Cotton and Milstein 1973) was described even before the seminal publication of monoclonal antibody technology (Köhler and Milstein 1975). This early experiment showing expression of both parental immunoglobulin genes in the hybrid cell was performed to better understand allelic exclusion, whereby under normal conditions each B lymphocyte produces antibodies encoded by only one of two possible alleles.

In the following years it became obvious that bsAb can be used to redirect immunological effector cells or molecules toward tumor cells. Targeting an immune response to the tumor site has evolved as an attractive concept since it recruits many effector cells and obviates several drawbacks connected with classical anti-tumor responses (reviewed by Fanger et al. 1992; Renner and Pfreundschuh 1995; van Spriel et al. 2000; Müller and Kontermann 2007a).

Basically, there are three methods by which bsAbs can be obtained. The first generation of bsAb was produced either by chemical coupling of different immunoglobulin Fab′ fragments at the hinge region (Glenie et al. 1987) or by cell fusion of two hybridoma cell lines, resulting in a quadroma cell which secretes among other immunoglobulin combinations also bsAb (Milstein and Cuello 1983).

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Although some of these early reagents showed remarkable efficacy in the treatment of certain malignancies, the vast majority of them did not met the high initial expectations because of toxicity and low therapeutic potency. Advance in genetic engineering has facilitated the creation of second-generation bispecific molecules of different sizes and binding strengths. These recombinant bispecific constructs paved the way for a revival of bsAb and renewed the interest in this novel type of modified antibodies (Little et al. 2000; Kufer et al. 2004; Weiner 2007; Müller and Kontermann 2007b, 2010; Chames and Baty 2009a, b; Beck et al. 2010; Chan and Carter 2010).

In this chapter, I describe the principles of hybrid hybridoma creation, give an overview on screening and purification procedures of bsAb, and finally outline the mechanisms by which this special class of antibodies exerts its effector functions. For the sake of clarity, I have restricted my viewpoint mainly to the field of cancer research and oncology/hematology.

2.2 Production of Hybrid Hybridoma by Cell Fusion

The major goal of fusing two permanently growing hybridoma lines is the selection of resulting hybrids and the exclusion of non-fused parental cells and intra-hybridoma fused cells. This can essentially be achieved by chemical selection procedures or by cell sorting techniques (Segal and Bast 2001).

2.2.1 Introduction of Selection Markers

In case of chemical selection, a mutation of the gene coding for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT) can be induced in one hybridoma line (Chervonsky et al. 1988). HGPRT-negative mutants can be selected by culturing the cells in the presence of toxic purine analogs like 8-azaguanine or 6-thioguanine. Cells harboring the enzyme are killed after incorporation of the toxic nucleotide. Spontaneously arising mutants can simply be established because the enzyme is encoded on the X chromosome and only one gene locus has to be targeted. Proliferation of the other parental line can be irreversibly inhibited by treatment with iodoacetamide just before hybridization (Gilliland et al. 1988). If the fusion mixture is cultured in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium) only hybrid hybridomas can actively grow. This is schematically depicted in Fig. 2.1. The combination of HAT sensitivity and resistance to the drug ouabain was also successfully applied (Link and Weiner 1993).

Alternatively, one hybridoma line is selected for two mutations, e.g., HAT sensitivity and resistance to neomycin (neo^r). For this, a HGPRT-deficient hybridoma is subsequently transfected by electroporation with a vector containing the neo^r

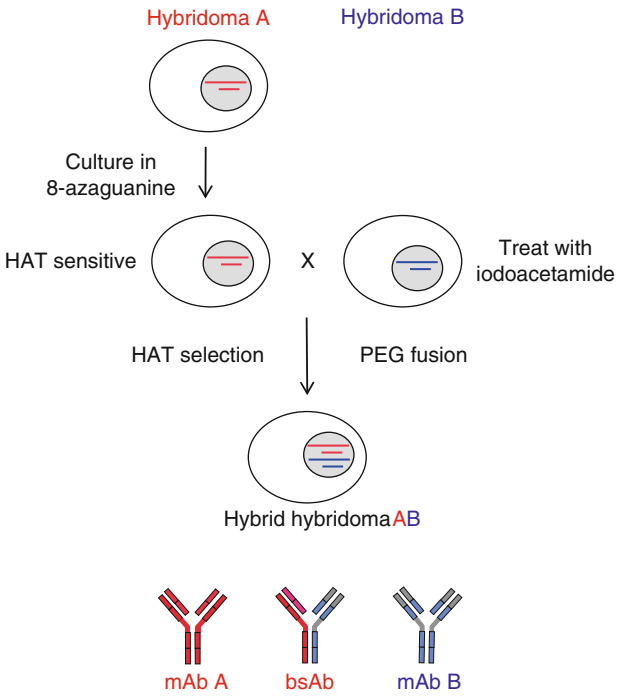

Fig. 2.1 Principle of hybrid hybridoma production by cell fusion. Sensitivity to HAT is induced in one hybridoma by selection with 8-azaguanine. Proliferation of the other hybridoma is inhibited by iodoacetamide treatment. After PEG fusion only hybrid hybridoma cells can actively grow in HAT medium

gene (De Lau et al. 1989). The advantage of such a double-mutant hybridoma line is that it can be fused with any unmodified second hybridoma line. As above, only hybrids can survive selection in medium supplemented with HAT and the neomycin analog G418.

2.2.2 Selection by Flow Cytometry

Fluorescence-activated cell sorting (FACS) offers another possibility to select hybrid hybridomas (Karawajew et al. 1987; Koolwijk et al. 1988). In this setting, one hybridoma cell line is labeled for instance with a green fluorochrome and the other with a red fluorescent marker. After cell fusion, double-labeled hybrid hybridomas are sorted and immediately cloned using a single-cell deposition device. Although this method is fast since it circumvents lengthy insertion of selectable markers there is a certain risk of contamination with parental cells.

2.2.3 Cell Fusion Procedures

For a long time polyethylene glycol is the fusion agent of choice (Pontecorvo 1975). It renders the membrane of cells to be fused gluey so that they will adhere together. Subsequently, plasma membrane fusion occurs giving rise to a cell with two (or more) nuclei. During cell division the nuclear membranes are degraded and the chromosomes are distributed into the daughter cell. These hybrid cells contain only one nucleus but the genetic material of both parents. Since hybridomas usually are hyperploid, a fusion of those will harbor a large excess of chromosomes that causes genetic instability during further mitoses leading to improper segregation or loss of chromosomes. To stabilize the chromosomal inventory of hybrid hybridoma repeated cloning by limiting dilution is inevitable. This also allows the selection of subclones producing high amounts of the desired bsAb.

As an alternative to the polyethylene glycol method, electrically induced cell fusion has been developed. It is based on the delivery of high-voltage electrical field pulses to physically fuse hybridoma cells (Cao et al. 1995; Kreutz et al. 1998).

2.2.4 Screening Hybrid Hybridoma Cultures for Specific Antibody

Establishment of a reliable, sensitive, and fast screening assay for the detection of desired bispecific antibody is the most important prerequisite for successful quadroma production. It is not recommended to start a fusion experiment before an appropriate screening assay has been set up. There are different test types available for the initial screening that all are based on the measurement of antigen–antibody binding. Enzyme-linked immunosorbent assay (ELISA), flow cytometry, and cytotoxicity are the most commonly used methods (Moldenhauer 2007). Choosing parental hybridoma cell lines of distinct isotype will facilitate the screening procedure tremendously.

Solid-phase ELISA (Engvall and Perlman 1971) where the antigen is immobilized on the well of a microtiter plate represents a universal test system that can easily be customized and allows rapid analysis of many samples in parallel. By ELISA one can simply detect bsAb with two different heavy chains. For this, the assay plate is coated with a catcher antibody specific for one isotype (e.g., mouse IgG1). Binding of bsAb is recognized by a detector antibody specific for the other isotype (e.g., mouse IgG2a). If one or both target antigens are available as proteins, they can be immobilized onto the assay plate. Again, reactivity with isotype-specific second step reagents is indicative for bsAb.

Bispecific antibodies developed for cancer therapy are usually directed against cell surface molecules on tumor and effector cells. Fluorescence-activated cell analyzer and cell sorter provide extremely valuable tools for the rapid, reliable, and quantitative screening of antibodies interacting with cell surface receptors.

Binding of bsAb to cells is monitored by isotype-specific fluorescently labeled secondary antibody. Thereby, the mean fluorescence intensity reflects the binding strengths of the respective antibody arm to its cellular target.

To test for bsAb-induced redirected cytotoxicity is a straight forward but technically demanding approach (Clark and Waldmann 1987). Target and effector cells are incubated in the presence of bsAb (and appropriate controls) leading to specific lysis of antigen-bearing targets. For the quantitative evaluation either the classical chromium release test (that requires initial labeling of target cells with radioactive chromium-51) or non-radioactive methods measuring the release of the endogenous cytosolic enzymes as for instance lactate dehydrogenase (Decker and Lohmann-Matthes 1988) or glyceraldehyde-3-phosphate dehydrogenase (Corey et al. 1997) may be applied. During the initial screening of culture supernatants (containing all immunoglobulin combinations) the signals obtained by flow cytometry and cytotoxicity test may be relatively weak which is caused by competition of parental monoclonal antibodies with bsAb for antigen binding.

2.2.5 Cloning

Rapid cloning of hybrid hybridoma cultures is mandatory to select for stable bsAb-secreting cell lines. The method of choice for single-cell cloning is limiting dilution. In principle, quadroma cells are distributed in 96-well plates so that one well will contain theoretically 0.5 or 1 cell. Usually the cloning efficiency of hybrid hybridomas is quite high and feeder cells are not required. Due to the genetic instability of hybrids it is possible to use repeated cloning steps to identify subclones with enhanced production of bsAb that can be verified by quantitative ELISA.

2.2.6 Purification of Bispecific Antibody

Once a stable hybrid hybridoma line is established, large quantities of antibody can be produced employing modern cell culture devices for long-term propagation. At least two systems are on the market meeting the demands of laboratory-scale production because they can simply be installed in a normal CO_2 incubator and do not require complicated pumping and other sophisticated equipment. Both, the miniPERM modular minifermenter (Falkenberg et al. 1995) and the two-chamber cell culture device CELLine 1000 (Trebak et al. 1999) are easy to handle and allow culturing of antibody-producing cells at high density (above 10^7 cells per ml). Harvest of the antibody-enriched product can be performed several times until productivity ceases.

Due to the huge variability in physicochemical properties of quadroma-derived IgG that largely depends (among other traits) from the chain composition no

standard procedure for purification can be recommended. In fact, the method has to be established for every individual bsAb. Only in rare cases the respective target antigens are available as (recombinant) proteins which can be employed for affinity purification (Gupta and Suresh 2002). Anti-idiotypic antibodies specifically interacting with the parental antibody were also employed but are very difficult to obtain (Bruynck et al. 1993). In retrospect, most investigators have used sophisticated two-step methods to purify bsAb from a mixture of mono- and bispecific immunoglobulin species. Often protein A or protein G affinity chromatography was combined with ion-exchange or size-exclusion chromatography (Tarditi et al. 1992). More recently, a hydrophobic interaction chromatographic technique was described that resolves bsAb, monospecific immunoglobulins, and culture medium supplements in one single step from bioreactor harvest (Manzke et al. 1997). The purity of bsAb can be assessed by SDS-PAGE, isoelectric focusing, Western blotting, isotype-specific ELISA or functional assays like T-cell proliferation.

2.2.7 *Molecular Composition of Bispecific Antibody*

So far, mostly mouse–mouse, rat–rat, and mouse–rat hybrid hybridomas have been created. Besides fusion of two IgG-secreting hybridomas also IgA-producing cell lines and hybrids between IgA and IgM were successfully established (Urnovitz et al. 1988). One major drawback connected with the production of bsAb by hybrid hybridoma is the co-dominant expression of immunoglobulin genes of both parents. This leads to random association of both parental heavy and light chains resulting in ten different antibody species only one of which constitutes the desired bsAb (Fig. 2.2). When assuming that the rate of synthesis is the same for all four chains and pairing happens randomly one would expect 12.5% of the total immunoglobulin to be bispecific (Staerz and Bevan 1986). Ideally, for a high yield of bsAb heterologous H–H chain pairing together with homologous H–L chain association

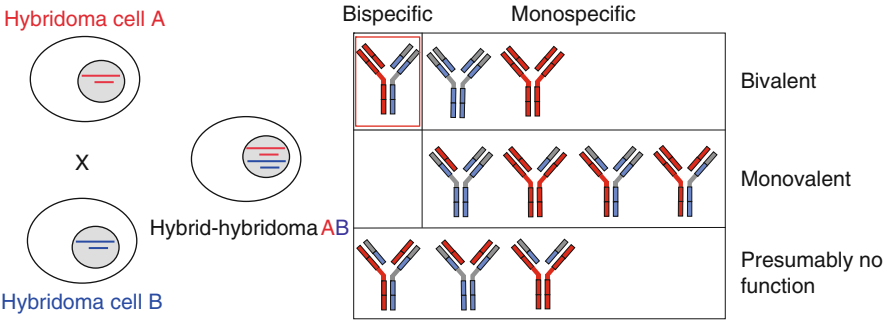

Fig. 2.2 Antibody species secreted by hybrid hybridoma. Immunoglobulin genes of both parental hybridomas are codominantly expressed in hybrid hybridoma leading to random association of heavy and light chains. Only one among the ten different antibody species is the desired bsAb

should occur. Unfortunately, this is not always achieved and preferential heterologous H–L chain association giving rise to antibodies with unknown specificity has been described (De Lau et al. 1991). The use of identical IgG isotype appears to favor mixed H–H chain combinations, whereas different isotypes lead to a reduced yield of bsAb (Milstein and Cuello 1984). Preferential species-restricted H–L chain pairing was observed in certain rat/mouse quadromas (Lindhofer et al. 1995). However, parental hybridomas of different IgG isotype offer great advantages with regard to purification since the resulting bsAb are bi-isotypic and can therefore be separated from parental immunoglobulins.

2.3 Functional Features of Bispecific Antibody

The key function of a bsAb for tumor therapy is of course the recruitment of immune effector cells to the tumor site. Depending on the type of effector cell, additional functional requirements have to be fulfilled. Under optimal conditions, the tumor cell will be finally destroyed by the common mechanisms of necrosis or apoptosis. A schematic representation of bsAb-mediated retargeting of various effector cells is given in Fig. 2.3.

BsAb from hybrid hybridoma display the same size (MW approximately 150 kDa) and high stability as conventional IgG antibodies. Thus, their serum half life and tissue penetration is comparable. To avoid interaction with Fc receptor-bearing cells and to increase tumor accessibility $F(ab')_2$ fragments were produced by enzymatic digestion (Warnaar et al. 1994). Fragmentation of bsAb is not an easy task and the protocol has to be adapted for each individual candidate to prevent complete degradation.

The avidity of both binding arms usually reflects the binding strengths of the parental antibodies at least under monovalent binding conditions in vitro.

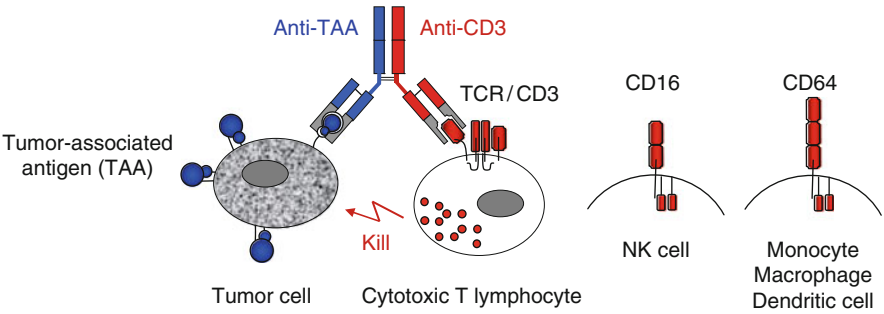

Fig. 2.3 BsAb-mediated retargeting of effector cells. BsAb binds with one arm to a triggering molecule on the effector cell and with the other arm to a tumor-associated antigen on the malignant cell. Conjugate formation of both cells results in lysis of the target cell. The main trigger molecules on cytotoxic T lymphocytes, NK cells, and myeloid cells are depicted

The actual avidity can be estimated by a flow cytometric binding competition assay. For this, increasing amounts of bsAb compete for binding to the antigen-bearing target cell with the fluorescently labeled parental antibody of known affinity. More accurate affinity measurements by surface plasmon resonance (BIACORE technology) are possible if the target antigens are available as (fusion) proteins (van Regenmortel 2003).

The innate and adaptive immune system contains distinct effector cell populations that can elicit cytotoxicity; they comprise T lymphocytes, natural killer cells, monocytes/macrophages, and polymorphonuclear neutrophils (PMN). These cell types carry specific trigger molecules on their cell surface that interact with natural ligands on the target cell. Binding of bsAb to the trigger molecule replaces the interaction with the ligand and leads to activation.

2.3.1 Recruitment of Cytotoxic T Lymphocytes

From the beginning, cytotoxic T lymphocytes were mostly used as effector cells for tumor attack as they represent the professional killers of the immune system (Liu et al. 1985; Perez et al. 1985; Staerz et al. 1985; Lanzavecchia and Scheidegger 1987). Under physiological conditions T cell specificity is determined by the T cell receptor (TCR) that recognizes peptides in conjunction with major histocompatibility complex (MHC) molecules. T cells need a second signal to become fully activated. This is delivered by the CD28 receptor on T cells that interact with costimulatory molecules of the B7 family exposed on antigen-presenting cells. Injected into the circulation, a bsAb binds only with one arm to a T lymphocyte that is not sufficient for triggering. Subsequently, if the bsAb-coated T cell finds and interacts with the target antigen expressing tumor cell functional crosslinking of the CD3/TCR complex occurs leading to a first step of activation. If costimulation is also provided at the tumor site either by CD28 ligation on the same T cell or secretion of lymphokines and cytokines by neighboring cells than cytotoxicity is fully established. This mechanism has been denoted as target cell-induced T cell activation (Jung et al. 1987; Jung and Müller-Eberhard 1988).

Retargeting of cytotoxic T cells versus tumor cells offers two fundamental advances. First, in contrast to conventional mono- or oligoclonal T cell activation by tumor-associated antigens bsAb containing an anti-CD3 moiety are able to induce a polyclonal T cell proliferation. Thus, a huge number of effector T cells are recruited to the tumor site. Secondly, bsAb-guided T cells also attack MHC-negative target cells because the specificity of the reaction is dictated by the antibody and not by the genetically determined TCR. This feature is of special importance in light of the fact that many tumors show reduced or even absent MHC expression as a consequence of dedifferentiation in the course of progression (Garrido and Algarra 2001). MHC loss represents an important tumor escape mechanism that potentially can be overcome by bsAb therapy. Antitumor cytotoxicity is mainly achieved by $CD8^+$ lymphocytes but in the human system also $CD4^+$ cells have the capacity to kill.

Tumor cell lysis following retargeting of T cells by bsAb engages the granule exocytosis system that relies on the directed release of granules containing perforin and granzymes from activated lymphocytes after specific recognition and conjugate formation with the target cell (Renner et al. 1997).

As mentioned above, full activation of effector T lymphocytes is an important prerequisite for the establishment of a robust cytotoxic reaction. This can be achieved via stimulation of the TCR/CD3 complex in combination with CD28 costimulation. Commonly, tumor cells are not able to provide costimulatory signals since they lack expression of members of the B7 family that interact with CD28 on the effector cell. Consequently, in many preclinical and clinical studies costimulation was made available by the additional application of conventional bivalent anti-CD28 monoclonal antibody (Manzke et al. 2001a; Manzke et al. 2001b) or by the use of two bsAb one of which is directed against CD28 (Bohlen et al. 1993; Pohl et al. 1993; Kroesen et al. 1995). In this setting both bsAb recognize the same target antigen but one reacts with CD3 and the other with CD28 to ensure activation (via CD3) and costimulation (via CD28) of T cells in a target cell restricted manner.

Depending on the epitope they bind on the CD3 coreceptor, some bsAb do not require further costimulation in order to induce a vigorous T cell activation. Although this strengthens the potency of therapeutic bsAb, at the same time it enhances the risk of adverse side effects caused by the release of lymphokines and cytokines. In the worst case a global T cell activation can cause a cytokine-release syndrome ("cytokine storm") that represents a life-threatening adverse event. Based on an incidence in 2006 following the application of a superagonistic anti-CD28 antibody in six human healthy volunteers with the development of unexpected severe adverse events including multiorgan failure (Suntharalingam et al. 2006) the German Paul-Ehrlich-Institut (regulatory authority for the approval of mAb) is classifying bsAb as high-risk drugs (Schneider et al. 2006).

To avoid unwanted side effects caused by excessive cytokine release, some investigators have administered bsAb in a locoregional fashion, e.g., by direct intratumoral or intralymphatic injection in case of low-grade B cell lymphomas (Manzke et al. 2001a; Manzke et al. 2001b). In a previous study we found that tumor-associated lymphocytes present in malignant ascites and pleural effusion from patients with ovarian and breast cancer are already in a preactivated state and able to efficiently lyse tumor cells in the presence of a bsAb of EpCAM × CD3 specificity without further costimulation (Strauss et al. 1999). These data provided the rationale for a pilot study of intraperitoneal bsAb therapy in patients with advanced ovarian cancer and malignant ascites (Marmé et al. 2002). Another possibility to minimize adverse reactions is to expand and to preload T lymphocytes from peripheral blood ex vivo with bsAb and to re-infuse them into the patient. This strategy representing a special form of adoptive immunotherapy was pursued in several studies of epithelial tumors leading to malignant effusions. BsAb-coated preactivated T lymphocytes were injected either alone or with additional soluble bsAb in the pleural or abdominal cavity of the patient (Bolhuis et al. 1992; Kroesen et al. 1993; Canevari et al. 1995). Lastly, the most demanding procedure is the systemic application of bsAb. A phase I study was conducted in patients with renal

cell cancer receiving increasing doses of $F(ab')_2$ fragments of an EpCAM × CD3 bsAb together with subcutaneous IL-2 (Kroesen et al. 1994). Although high serum levels of pro-inflammatory cytokines were noted in the patients, the feasibility of this approach could be demonstrated. Further clinical experience was reported in a dose escalation study applying a CD19 × CD3 bsAb in non-Hodgkin's lymphoma patients (de Gast et al. 1995). Noteworthy, only limited toxicity (WHO grade II) occurred which was mainly attributed to tumor necrosis factor alpha release.

Intact bsAb carry a Fc portion that potentially can bind to Fc-receptors on a variety of accessory cells. Mouse bsAb composed solely of IgG1 or a combination of IgG1 and IgG2a heavy chains are poor mediators of ADCC in human effector cells due to their weak interaction with activating Fcγ receptors like CD16 and CD64. To improve binding to Fc-receptors, so-called trifunctional bsAb consisting of mouse IgG2a and rat IgG2b and directed against CD3 and a tumor-associated antigen were created. In addition to their dual specificity via the two binding domains, these reagents are able to efficiently recruit and activate accessory cells as macrophages, NK cells, and dendritic cells by their Fc region leading to secretion of various cytokines. This third function is able to provide the essential costimulatory signals to cytotoxic T-lymphocytes (Zeidler et al. 1999). Importantly, most likely by phagocytes ingesting tumor material a long-lasting anti-tumor immunity is achieved (Zeidler et al. 2000; Ruf and Lindhofer 2001). One trifunctional bsAb named catumaxomab (Removab®) with specificity for the epithelial cell adhesion molecule (EpCAM) and CD3 has proven to be especially effective for the intraperitoneal treatment of malignant ascites which develops during advanced stages of a variety of intra-abdominal malignancies as ovarian and stomach cancer (Burges et al. 2007; Heiss et al. 2010). As first bsAb worldwide catumaxomab was approved in April 2009 in the European Union (Seimetz et al. 2010) and will be discussed in a separate chapter of this book.

In addition to the TCR-associated CD3 molecule other T cell antigens as CD2 (Wild et al. 1999), CD5, and the TCR itself (Ferrini et al. 1989) have been engaged as trigger molecules of bsAb. Our group has recently reported on a new quadroma-derived bsAb of the specificity CD19 × CD5 that was employed to target an ex vivo expanded and activated T cell subset called cytokine-induced killer (CIK) cells expressing CD5 (Tita-Nwa et al. 2007). Importantly, CD5 targeting bsAb may be particularly useful in combination with adoptive T cell transfer, e.g., in the setting of allogeneic stem cell transplantation, as it neither activates nor induces proliferation of naïve T cells potentially directed against host antigens. Thus, the danger of a graft versus host reaction in the recipient might be diminished.

But what actually happens when a target cell and an effector cell encounter each other in the presence of bsAb? We have addressed this question for a bsAb with EpCAM × CD3 specificity in two experimental systems. In the first setting a collagen gel three-dimensional tumor reconstruct was used which closely resembled the tumor microenvironment. Dynamic tumor cell–lymphocyte interactions were recorded by time-lapse video microscopy. Contact duration was about three times longer in the presence of bsAb compared with control Ab, whereas lymphocyte velocity was not influenced (Salnikov et al. 2009). This finding indicates that

bsAb facilitates and prolongs interactions between tumor cells and lymphocytes regardless of TCR specificity. In a second set of experiments the molecular binding characteristics of the EpCAM × CD3 bsAb were analyzed by Single Cell Force Spectroscopy using an atomic force microscope. The investigation reveals that bsAb-induced conjugate formation between T cells and tumor cells occurs in a biphasic process that finally leads to the development of a complete immune synapse. The early phase of cell adhesion is specific and reversible and consecutively adhesive forces increase in a time and contact force-dependent manner (Hoffmann et al. 2011). It appears likely that extended contact time and increasing binding forces between effector and target cell will ultimately initiate T cell activation and signaling as indicated by the formation of a mature synapse.

2.3.2 Recruitment of Fc Receptor-Bearing Effector Cells

Besides T lymphocytes, Fc receptor-carrying cells of the innate immune system have been extensively exploited as effectors. Natural killer cells, monocytes, macrophages, dendritic cells, and neutrophils expose (either constitutively or upon induction with cytokines) Fc receptors on the surface that can be engaged by bsAb. In general, two classes of Fc receptors can be distinguished according to their function: activating and inhibitory receptors that differ with respect to their cytosolic signal transduction pathway. For bsAb-mediated targeting strategies especially the activating receptors FcγRIA (CD64), FcγRIIA (CD32), FcγRIIIA (CD16), and FcαRI (CD89) are of interest. Nevertheless, one important characteristic of the Fc receptor system is the coexpression of activating and inhibitory Fc receptors on the same cell whereby inhibitory signaling dominates activation in order to prevent unspecific stimulation of the immune system (Nimmerjahn and Ravetch 2006; Nimmerjahn and Ravetch 2007). Natural killer cells represent an exception from this rule since they carry only the activating FcγRIIIA (CD16). Functionally, they are cytotoxic lymphocytes which without prior stimulation can kill a variety of target cells in a non-MHC-restricted fashion. They belong to the first line of cellular defense of the innate immune system. Ligation of CD16 by bsAb is able to activate resting NK cells and induce lysis of tumor cells. Under certain conditions additional application of IL-2 can even enhance the efficacy (Ferrini et al. 1992; Hombach et al. 1993). First clinical studies evaluating NK targeting via CD16-specific bsAb showed some minor responses that were achieved at the expense of severe adverse reactions.

Cells of the myeloid lineage constitutively express activating Fc receptors that have been employed as exquisite trigger molecules for the induction of cytotoxicity. Especially the high affinity receptor for IgG, termed FcγRIA or CD64, was identified as a well-suited target molecule for bsAb-based immunotherapy of cancer (Deo et al. 1997; van Spriel et al. 2000; Schweizer et al. 2002). CD64 constitutes an activating Fc receptor on monocytes, macrophages, and dendritic cells, whereas its expression can be induced on neutrophils and eosinophils by IFN-γ or G-CSF

treatment. This is of special relevance since polymorphonuclear neutrophils (PMNs) are the most abundant circulating white blood cells and able to elicit strong cytolytic and phagocytic activities. They release soluble chemotactic factors that recruit further nonspecific and specific immune effector cells. Importantly, induction of FcγRIA on granulocytes and substantial upregulation on macrophages can be easily achieved in patients by systemic application of the respective cytokine or growth factor (Repp et al. 1995).

Several bsAb recognizing the CD64 molecule in conjunction with an epithelial tumor-associated antigen were established during the past years. The tyrosine kinase receptor HER2/neu, the EGF receptor, the EpCAM cell adhesion molecule, and the MUC1 antigen are prominent examples for the second binding specificity for those bsAb being prepared or having already entered clinical testing. So far, most bsAb trials taking advantage of FcγRIA targeting have demonstrated moderate to low toxicity but a sustained tumor regression was achieved only in few patients (Curnow 1997; James et al. 2001). Dendritic cells that also express Fc receptors were shown to efficiently take up dying antibody-coated tumor cells. This might lead to an enhanced cross presentation and generation of tumor-specific T cell responses and ultimately boost antitumor immunity (Weiner et al. 2009). Finally, in recent studies the Fc receptor for IgA, CD89, has attracted much attention for bsAb-guided therapy. Comparison of different trigger molecules expressed on neutrophils revealed that CD89 is the most potent Fc receptor for tumor cytolysis (Valerius et al. 1997).

2.3.3 Human Anti-Rodent Immunoglobulin Response Following Bispecific Antibody Therapy

One of the problems related to the therapeutic application of a murine or rat bsAb is the occurrence of a human anti-mouse antibody (HAMA) or human anti-rat antibody (HARA) response. It has been shown that HAMA/HARA production may lead to allergic reactions ranging from mild cutaneous eczema to anaphylactic shock (Khazaeli et al. 1994). Apart from inducing adverse events, HAMA was described to block the binding of bsAb to its targets and thereby inhibit cytotoxicity toward the tumor cells (Lamers et al. 1995). Whether HAMA directed against the constant part of the mouse immunoglobulin molecule or merely anti-idiotype antibodies is responsible for such an inhibitory effect in patients has to be further investigated. In a retrospective study data from three different clinical trials employing $F(ab')_2$ fragments of mouse bsAb of the specificity CD3 × folate receptor for the intraperitoneal treatment of ovarian cancer were analyzed. As one parameter, the influence of HAMA response on the therapeutic efficacy of the murine bispecific antibody was evaluated. Surprisingly, patients with high HAMA levels had a significantly longer median survival probability than patients with a minor HAMA response (Miotti et al. 1999). Similar results were reported

from a study using conventional bivalent mouse monoclonal antibodies to treat B cell lymphoma patients (Azinovic et al. 2006).

There are at least two explanations for this unexpected phenomenon. It is well established that mouse antibodies can induce a significant anti-idiotypic response in humans especially after multiple applications. According to the network hypothesis, the anti-idiotype antibody is immunogenic itself and induces a humoral immune response by the formation of anti-anti-idiotypic antibodies. Since it is believed that the anti-idiotype mimics an epitope of the antigen the initial antibody binds to (the anti-idiotype represents an internal image of the antigen) this immune response is not only directed against the anti-idiotypic antibody but at the same time against the primary target. In addition, complexes containing the murine therapeutic antibody and the human anti-idiotype are internalized and presented by professional antigen presenting cells. Through the presentation of internal image peptides on MHC molecules T cells might be stimulated which subsequently elicit an anti-tumor response. A second possible mechanism may be the direct induction of a cellular immune response by the murine antibody through its MHC-complexed presentation by antigen presenting cells. In this case, the processed mouse antibody presented on the tumor cell would serve as a new artificial tumor-associated antigen that could be recognized by cytotoxic T cells. In a previous investigation we found that tumor patients treated with mouse mAb develop T cells that recognize processed murine immunoglobulin on autologous antigen presenting cells in a MHC class II-restricted fashion. Mouse mAb directed against various cell surface molecules can thus be used as antigens to focus these T cells against an MHC class II positive target of choice (Lanzavecchia et al. 1988). A cellular immune response would certainly explain the efficacy of an antibody therapy even after formation of neutralizing HAMA.

2.4 Conclusion and Outlook

The past 25 years of bsAb development have seen several excitements and drawbacks. Nevertheless, the idea to use redesigned antibody molecules for the recruitment and retargeting of immunological effector cells to the tumor site has retained its attraction. One recent milestone was the approval of the first bsAb catumaxomab in Europe for treatment of malignant ascites arising from EpCAM-expressing carcinomas (Heiss et al. 2010; Seimetz et al. 2010). Another highlight was the impressive clinical results obtained with the recombinant CD19 $\times$ CD3 bispecific T cell engaging (BiTE) construct blinatumomab in patients suffering from non-Hodgkin's lymphoma (Bargou et al. 2008; Baeuerle and Reinhardt 2009).

A multitude of bispecific therapeutics is currently under clinical evaluation. New applications of bsAb will be further investigated as targeting of two independent receptors on the surface of one cell or pre-targeting strategies to improve chemo- and radiotherapy (Müller and Kontermann 2010). The induction of specific and long-lasting antitumor T cell responses seems to be feasible by enhancing the

capacity of bsAb to engage professional antigen-presenting cells via Fc receptors (Weiner et al. 2009). Moreover, bsAb hold great promise to guide gene-modified effector cell populations (e.g., TCR-modified T lymphocytes) in order to increase specificity and affinity in adoptive cellular therapies. After all, there are many good reasons to believe that now we shall see the light at the end of the tunnel of bsAb-based cancer therapy in near future (Chames and Baty 2009a).

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