Bispecific Antibodies: Molecules That Enable Novel Therapeutic Strategies

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Abstract
Bispecific antibodies are unique in the sense that they can bind simultaneously two different antigens. This property enables the development of therapeutic strategies that are not possible with conventional monoclonal antibodies. The large panel of imaginative bispecific antibody formats that has been developed reflects the strong interest for these molecules. Although in many cases the manufacturing of clinical grade material remains challenging, several bispecific antibody formats are currently in clinical trials.

Introduction
Since the end of the 19th century, antibodies have raised many hopes for the development of novel therapies. Although they may not always have been the ‘magic bullets’ that Paul Ehrlich had predicted, they are currently the fastest growing class of therapeutic molecules. Indeed, 18 monoclonal antibodies (mAbs) have so far been approved by the Food and Drug Administration (FDA) and more than 150 are at different stages of clinical development [1]. This success and enthusiasm in the drug development community can be largely explained by the properties of antibodies: their exquisite binding specificity and their low intrinsic toxicity as they are naturally present at a significant concentration in blood. Since the first description of a mAb-producing hybridoma [2] tremendous efforts have led to the development of new technologies to produce and engineer antibodies with desired properties. It is now possible to generate mAbs to virtually any antigen by using hybridoma or various antibody display technologies [3, 4]. Furthermore the immunogenicity observed with mouse or early chimeric antibodies can also be reduced by humanizing antibodies or deriving fully human antibodies directly from human genes [1]. Antibodies exert their therapeutic effect mainly by two mechanisms: (1) neutralizing the activity of the target antigen by binding to it (e.g. toxin, pathogen, receptor or ligand) and (2) they serve as targeting molecules for a toxic moiety or an effector function (e.g. drug, prodrug, radionuclide, antibody-dependent cell-mediated cytotoxicity or complement-dependent cytotoxicity).

In its classical IgG format, an antibody is a bivalent and monospecific molecule (fig. 1a). However, it was recognized very early during hybridoma technology development that hybrid hybridomas could be created by a fusion event between two hybridomas [5–7]. These so-called ‘quadromas’ expressing two different heavy and two different light chains produce a variety of different antibody species resulting from the random pairing of the heavy and light chains. From this process, bispecific antibodies (bsAbs) are generated, carrying a different specificity on each arm. The existence of these novel molecular structures has opened completely new therapeutic perspectives, leading to the development of technologies aimed at producing bsAbs in a rational and efficient manner.
In this review we will describe the main formats of bsAbs that have been explored, discuss novel therapeutic strategies that these molecules make possible and highlight ongoing clinical trials conducted with bsAbs.

**Technologies to Generate bsAbs**

**Chemical Cross-Linking***

A direct and straightforward approach is the use of chemical cross-linking reagents to covalently link two antibodies. In most cases, F(ab’)_2 fragments are generated from the parent antibodies by enzymatic digestion. Upon reduction of the inter-heavy chain disulfide bonds, F(ab’) that contain exposed thiol groups are generated. The latter are the main site of modification for homobifunctional reagents that allow for heterodimer assembly by random association of the F(ab’) fragments (fig. 1b) [8, 9]. The development of heterobifunctional reagents that activate one protein and allow it to react with a second has greatly increased the efficiency of bispecific product formation [10]. The major problem associated with chem-

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**Fig. 1.** Schematic representation of various bsAb formats. **a** Structure of a normal IgG molecule. **b** Bispecific F(ab)_2 obtained via chemical cross-linking. **c** bsAb fragments. **d** Bispecific formats obtained by fusing antibody fragments to constant domains (Fc or CH3). **e** Bispecific formats using other protein-protein interaction domains (leucine zippers or PKA-AKA interaction domains). **f** Bispecific IgG-like formats (knob-into-hole; bsAb from quadrroma; single domain bsAb). sdAb = Single domain antibody.
Bispecific Antibodies

Recombinant bsAbs

The development of genetic engineering has paved the way for the generation and evaluation of a very large number of bsAb fragments and whole IgG formats. It is beyond the scope of this review to discuss them all but we will focus on key representative examples.

A single-chain Fv (scFv) is an antibody fragment in which VH and VL domains are joined by a flexible linker that forces them to assemble in a stable manner despite the limited interaction surface [13, 14]. Clearly, scFv have been a major source of inspiration for bsAb engineering. First, it was demonstrated that two scFv could simply be joined by an additional linker to obtain a bivalent bispecific scFv also called a tandem scFv (tascFv) (fig. 1c). Although some tascFv have been expressed in *Escherichia coli*, they often appear to be poorly soluble and therefore have to be refolded from inclusion bodies or expressed in mammalian cells [15–18].

Another way to generate a bsAb using a scFv is to shorten the linker separating the VH and VL so that these domains cannot assemble and are forced to pair with the VH and VL from a second polypeptide thus generating a bivalent molecule called a diabody (Db) [19]. Bispecific diabodies can be assembled if the VH of a first antibody linked to the VL of a second antibody is coexpressed with the VH of the second antibody fused to the VL of the first antibody (i.e. VHa-VLb; VHb-VLa; Db; fig. 1c) [20]. The crystal structure of diabodies suggests a large degree of flexibility [21, 22]. Thus the two combining sites located at the opposite ends of the molecule are able to reach their target antigens if, for example, they are located on two different cells. However, it is likely that tascFv have a larger degree of flexibility, which might be important for binding epitopes that are less accessible. From a manufacturing perspective, diabodies are very attractive as they can be expressed at high levels in bacteria or yeast [23]. However, even if the correct VH-VL pairing should favor heterodimer formation, homodimers can also be formed leading to heterogeneity. By further linking two diabodies, tandem diabodies (taDb) can overcome this problem (fig. 1c) [16]. Interestingly taDb maintain the favorable expression properties and solubility of diabodies as they can be expressed as monomers in bacteria. Furthermore, taDb also appear to be more stable in vivo when compared to Db or tascFvs [24, 25].

A major limitation of antibody fragments is their short serum half-life due to their small size and lack of interaction with the neonatal Fc receptor as these constructs do not carry an Fc domain [24]. Attachment of polyethyleneglycol is a way to increase the size and improve the pharmacokinetic properties of molecules and this approach has been used successfully for scFvs and a number of therapeutic proteins [26, 27]. It should also be possible to pegylate bsAb fragments. The addition of an Fc domain to an antibody fragment is a clear alternative to increase serum half-life and several formats of such ‘mini-bodies’ have been described. These include Db-Fc fusion, taDb-Fc fusion or taDb-CH3 fusions [28, 29] (fig. 1d). These molecules are tetravalent with two binding sites for each antigen. Furthermore, an (scFv)₄-Fc molecule can be generated by fusing a first scFv to the CH1-CH2-CH3 domains of an IgG heavy chain and a second scFv to the CL domain of the light chain. The resulting IgG-like molecule bears a full Fc, is larger than an IgG and has four independent binding sites, two for each antigen ((scFv)₄-Fc; fig. 1d) [30]. As in principle no mispairing between chains can occur, this format does not present any heterogeneity problems. The drawback to date is its expression level which is currently an insufficient yield to allow its evaluation in animal models of human diseases [29].

The use of linkers is not limited to connecting domains that are derived from an IgG. Other multimerization domains, such as leucine zippers, have been used to force two binding specificities into a single molecule (fig. 1e) [31, 32]. Recently the interaction between the regulatory subunit of cAMP-dependent protein kinase A (PKA) and the anchoring domain of A kinase anchoring proteins (AKA) has been used to connect two different Fab fragments. As the regulatory subunit of PKA dimerizes to interact with the anchoring domain, the resulting fusion molecule is both bispecific and trivalent (fig. 1e). Elegantly, cysteine residues were engineered at the extremities of the interacting domains so that the resulting trimeric Fab is stabilized by disulfide bridge formation. This format appears to be stable in serum and was used for in vivo imaging experiments [33].

While it is obvious that linkers have advantages for the engineering of bsAbs, they may also cause problems in therapeutic settings. Indeed, these foreign peptides might elicit an immune response against the linker itself or the junction between the protein and the linker. Further-
more, the flexible nature of these peptides makes them more prone to proteolytic cleavage, potentially leading to poor antibody stability, aggregation and increased immunogenicity. Thus ideally, one should aim at developing bsAbs that are very similar to IgG with minimal deviation from human sequences (fig. 1f). A very elegant approach coined ‘knob into hole’ aims at forcing the pairing of two different IgG heavy chains by introducing mutations into the CH3 domains to modify the contact interface. On one chain bulky amino acids were replaced by amino acids with short side chains to create a ‘hole’. Conversely, amino acids with large side chains were introduced into the other CH3 domain, to create a ‘knob’. By coexpressing these two heavy chains, more than 90% heterodimer formation was observed (‘knob-hole’) versus homodimer formation (‘hole-hole’ or ‘knob-knob’) [34]. The percentage of heterodimer could be further increased by remodeling the interaction surfaces of the two CH3 domains using a phage display approach and the introduction of a disulfide bridge to stabilize the heterodimers [35]. Although this format appears very attractive, no data describing progression towards the clinic are currently available. One constraint of this strategy is that the light chains of the two parent antibodies have to be identical to prevent mispairing and formation of inactive molecules. Although this might seem a major drawback, phage display libraries with limited VL diversity greatly facilitate the isolation of antibodies that share a common light chain but have different specificities [36]. In such antibodies, it is reasonable to expect that most of the binding specificity is conferred by the VH domain. Interestingly, it was observed that mouse single VH domains can be sufficient for specific binding to a target protein [37]. The fact that the immune systems of camelids (lamas and camels) and cartilaginous fish (nurse sharks) use single V domains fused to an Fc reinforced the concept that a single domain can be sufficient to confer high affinity binding [38, 39]. Cameld, shark and even human V domains are being developed as alternatives to antibodies but they also have implications for bsAb generation [40]. Indeed having a binding specificity defined by a single domain (VH or VL) allows for the generation of a new range of formats including a classical IgG in which each arm has the potential to bind two targets either via its VH or VL domain (single domain antibody; fig. 1f) [41]. This single domain IgG should have similar biochemical properties as a monospecific IgG and consequently solve many of the problems linked to bsAb production and heterogeneity. It is however likely that in most cases both antigens will not be able to bind simultaneously to each antibody arm due to steric hindrance. Furthermore it will not be possible to ensure that the antibody will bind a different target on each arm; thus the range of applications of such bsAbs will be limited.

**Quadromas**

By fusing either two hybridomas or one hybridoma with a B lymphocyte, it is possible to generate quadromas and triomas, respectively [5, 6]. These chimeras express two heavy and two light chains that assemble randomly thus leading to the generation of 10 antibody combinations. The desired bsAbs represent only a small fraction of the secreted antibodies and can be purified, providing the biochemical properties of the bsAb are sufficiently different from the other species. This purification step, often requiring a combination of chromatographic techniques, dramatically reduces production yields and increases manufacturing costs. More efficient purification strategies have been developed such as thiolphilic interaction chromatography or the use of hydroxylapatite columns to improve the purity of the bsAb [42, 43]. The generation of stable quadromas has also been optimized by introducing different selection markers into each hybridoma before fusion. Quadromas can then be selected in medium that contains both drugs [44, 45]. Alternatively, cells can be fluorescently labeled before fusion and the double-labeled quadromas can be isolated on a fluorescence cell sorter [46].

Therapeutic bsAbs isolated from quadromas are necessarily of rodent origin and this can lead to major immunogenicity issues that will probably limit the general use of quadromas in the future. However, lessons learned from these hybrid hybridomas may be applied for the generation of stable cell lines coexpressing humanized or human antibodies. Moreover, most recombinant or chemical approaches for the generation of bsAb require purification of the bsAb from a more or less complex mixture and the experience gained during the development of purification procedures for quadromas will be of considerable benefit.

**Therapeutic Strategies Enabled by bsAbs**

**Simultaneous Inhibition of Two Targets**

mAbs as a monotherapy have been used with considerable success for the treatment of various diseases and there are strong indications that combinations of antibody-based drugs may provide greater efficacy with minimal side effects in certain pathologies. For example, for...
the treatment of hepatitis C, XTLbio is currently evaluating XTL-6865, a combination of two fully human mAbs against the hepatitis C virus E2 envelope protein, in a phase Ia clinical trial in patients with chronic hepatitis C [47]. The rationale is that a combination of two antibodies that bind to different epitopes is essential to provide broad coverage of virus quasispecies and to minimize the possibility of escape from therapy. In the treatment of cancer, Genentech has already moved several mAb combination therapies into clinical trials. The effectiveness of combined treatment with trastuzumab (Herceptin, anti-HER2 receptor) and bevacizumab (Avastin, anti-VEGF antibody) is currently being evaluated in patients with HER2-positive metastatic breast cancer [48]. In vivo experiments suggest that combined blockade of the HER2 receptor and VEGF results in superior antitumor efficacy compared with either treatment alone, illustrating how targeting two distinct processes key to tumor progression, that is cell proliferation and angiogenesis, may have a therapeutic advantage [49]. Along the same lines, the National Cancer Institutes’ Cancer Therapy Evaluation Program is running a randomized phase III trial coadministering cetuximab (Herbitux, anti-EGF receptor) and bevacizumab (Avastin, anti-VEGF) together with combination chemotherapy in treating patients with metastatic colorectal cancer. Immunomedics is evaluating the effect of treatment with epratuzumab (Lympho-cide, anti-CD22) and Rutixan (rituximab, anti-CD20) on recurrent or refractory non-Hodgkin’s lymphoma (NHL), a B cell lymphoma. Their rationale is based on targeting two B cell differentiation markers on the lymphoma, which may induce different mechanisms of action and thereby have additive or synergistic antilymphoma effects. To date, the combination therapy is well tolerated and results in the disappearance or reduction of detectable cancer in a number of patients with NHL [50].

Clearly combining antibody-based drugs makes sense in particular clinical scenarios, and this approach has already proven its worth in early clinical trials. Nevertheless, there are major hurdles to overcome before such combination therapy becomes standard clinical practice. Not least is the issue of intellectual property: such combination trials are relatively easy to conduct when both antibodies are FDA-approved (18 antibodies so far) and are owned or licensed by a single company. However, the situation becomes complicated when the antibodies are owned by different, perhaps competing, companies. Another major issue is the economic consideration: the high costs involved in research, manufacturing and regulatory affairs in mAb development remain a major obstacle.

In the light of these drawbacks, an emerging alternative is the use of bsAbs for the simultaneous inactivation
of two target antigens with a single antibody. Synergistic effects may be expected when components involved in parallel signaling pathways are targeted (fig. 2a). Such strategies should be of particular interest for redundant signaling networks where one component can substitute for another and therefore limit the therapeutic efficacy of a single drug. Two formats of bsAbs targeting epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR) have been developed for the treatment of cancers [29, 30]. These receptors have been implicated in the tumorigenesis of several human cancers and evidence that IGFR expression can compensate for EGFR inhibition suggested that targeting both receptors would be beneficial and limit the appearance of resistant tumor cells escaping by receptor downregulation [51]. In one case, a tetravalent IgG-like molecule was constructed by fusing a different scFv to the heavy and light chains, whereas the other format was a Db-Fc fusion (fig. 1d). These studies show that both bispecific formats retained the activity of the antibodies from which they were derived as they blocked EGFR and IGFR signaling and mediated antibody-dependent cellular toxicity on tumor cells expressing these receptors. Animal models of human xenograft confirmed that simultaneously targeting both receptors was more effective compared to monotherapies with antibodies against either receptor [29, 30]. Although encouraging, the data also highlighted the difficulties encountered with expression and in vivo stability of these particular bispecific formats. The (scFv)₄-Fc fusion molecule appears to be expressed at very low levels preventing its use in animal models [30]. On the other hand the well-expressed Db-Fc fusion quickly lost binding activity in vivo probably due to dissociation of the noncovalently associated chains [29].

Retargeting

As most bsAb formats are capable of binding two molecules simultaneously, they are perfect bridging molecules. The majority of bsAb developed so far aim to selectively target cytotoxic effector cells or cytotoxic agents to cells involved in a disease process (fig. 2b). For applications in oncology, one of the antibody’s specificities has been directed against markers of tumor cells such as CD19, CD20, HER2, carcinoembryonic antigen (CEA), or epithelial cell adhesion molecule (EpCAM) [52–56]. As neovascularization is essential for solid tumor growth, markers of vasculature are also attractive targets as they are readily accessible to circulating therapeutic agents thus avoiding problems linked to drug tissue penetration [54]. The second arm of the bsAb is used to bring in close proximity a toxic moiety or activity. Drugs such as doxorubicin have been targeted to CEA-expressing colon cancer cells leading to inhibition of tumor growth in vivo and lowering the dose of drug required to reach half maximal inhibitory activity [57]. Toxins, cytokines and radiolabeled haptens have limitations such as short serum half-lives and intrinsic toxicity or systemic effects that can be improved by selective targeting with bsAb. Particularly promising are two-step strategies in which the bsAb is administered first allowing for tissue pretargeting, followed by radionuclide administration leading to high tumor-to-blood ratios and lowering general toxicity of radiotherapy [58, 59]. Along the same concept, antibody-directed prodrug therapy allows for the targeting of an enzyme that catalyzes the conversion of nontoxic molecules into toxic drugs in the vicinity of the target cells [60].

Strategies involving the activation and targeting of effector cells from the immune system to tumors have extensively been explored and some have advanced into clinical development. In order to recruit immune cells, one of the specificities of bsAb is directed against CD3 (T cells), CD16 (NK cells), CD64 or CD89 (monocytes and neutrophils). With the exception of CD89, engagement of the activating receptors by these bsAb is not sufficient to achieve tumor killing. Pre- or costimulation of effector cells is required [61–63]. In addition, relatively high doses of bsAb and high effector to tumor cell ratios are needed to obtain efficient tumor cell lysis, conditions that are not easily achieved in vivo [reviewed in 64]. Interestingly, a tascFv format referred to as bispecific T cell engager (BiTE), which has a very short linker (5 amino acids) between the two scFv, appears to have improved antitumor properties (fig. 1c). For example, a BiTE directed against CD19 and CD3 is able to mediate lysis of CD19-expressing tumor cells in the absence of pre- or costimulation [52]. Potentially, the smaller size of the BiTE brings the two cell types in closer contact and allows for an immunological synapse to form promoting strong activation of cytotoxic T cells [65].

Increased Avidity or Specificity

Antibody binding in a classical IgG format is directed by the affinity of each combining site for its antigen but also relies on avidity effects linked to bivalent binding. The latter dramatically increases the apparent affinity of the antibody for cell surface markers as two dissociation events have to occur for the antibody to be released. As shown above, some bispecific formats are bivalent (i.e. 8 Pathobiology 2007;74:3–14

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one binding site for each target) whereas others are trivalent or tetravalent (fig. 1). The latter have the potential to bind each target in a bivalent manner although steric hindrance could limit this possibility. Both format types have advantages depending on the therapeutic application. For instance, cross-linking of a receptor is often a prerequisite for antibody-mediated activities such as signaling, internalization or apoptosis and can only be achieved if the receptor is bound by both arms of the antibody. Therefore, higher affinity and avidity would appear beneficial for many therapeutic applications. However, it must be considered that high affinity antibodies are mainly localized at the periphery of solid tumors potentially lowering antitumoral activity [66, 67]. The large size of IgG certainly limits diffusion into the tumor, but additionally high affinity binding also restricts tumor penetration as these antibodies stay bound to antigens first encountered in the tumor periphery. Indeed, using a panel of tumor targeting scFv with different affinities, peripheral localization correlated with higher affinity [68]. In this context, bsAb formats with monovalent antigen binding and appropriate affinity might have better tumor penetration properties.

It is also possible to generate bsAb that bind in a monovalent manner two tumor antigens thus requiring the presence of both antigens on a cell to benefit from avidity and slow dissociation resulting in increased specificity towards cells expressing both markers, relative to cells expressing only one antigen [69] (fig. 2c). This is of a particular importance as most tumor markers are only upregulated on tumor cells and are also present on other cell types. Thus, lower avidity binding to nontumor cells can limit unwanted toxicity.

### bsAbs in the Clinic

Up until recently, laborious production procedures have considerably hampered the clinical evaluation of bsAbs. These production issues are still a major concern but alternative recombinant strategies have rendered possible the expression of particular bsAbs formats to reasonable yields and their purification to homogeneity, suggesting that drug supply will no longer be a limiting factor. Several bsAbs have entered clinical trials (table 1) and some studies have generated encouraging data where-

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**Table 1. bsAb recently in clinical trials**

<table>
<thead>
<tr>
<th>Name</th>
<th>Company</th>
<th>Species</th>
<th>Target</th>
<th>Format</th>
<th>Purification</th>
<th>Latest stage of clinical development</th>
</tr>
</thead>
<tbody>
<tr>
<td>MT103 (MEDI-538)</td>
<td>Micromet MedImmune</td>
<td>Murine</td>
<td>CD19 × CD3</td>
<td>BITE Tandem single chain (VL-VH)CD19-L-(VH-VL)CD3-6-His ( L = 5 ) amino acids</td>
<td>His-tag</td>
<td>Phase I: preliminary results of 19 patients; non-Hodgkin lymphoma</td>
</tr>
<tr>
<td>HD37 × TR66</td>
<td>TRION Pharma Fresenius Biotech GmbH</td>
<td>Murine</td>
<td>HER2/neu × CD3</td>
<td>Intact IgG (quadroma)</td>
<td>Protein A</td>
<td>Phase II/Ill: malignant ascites Phase II: metastatic breast cancer</td>
</tr>
<tr>
<td>Catumaxomab Removab™ C215 × 26I16</td>
<td>Micromet MedImmune</td>
<td>Murine</td>
<td>CD19 × CD3</td>
<td>Tandem single chain (VL-VH)CD19-L-(VH-VL)CD3-6-His ( L = 18 ) amino acids</td>
<td>Protein L</td>
<td>Phase I completed: 17 patients; metastatic melanoma</td>
</tr>
<tr>
<td>Erturnoxomab Remun™ 2502A × 26I16</td>
<td>TRION Pharma Fresenius Biotech GmbH</td>
<td>Murine</td>
<td>HER2/neu × CD64</td>
<td>Fab’ chemical conjugate</td>
<td>Gel filtration chromatography</td>
<td>Phase I/II completed: 30 patients; advanced breast cancer</td>
</tr>
<tr>
<td>rM28 9.2.27 × 9.3</td>
<td>University of Tübingen</td>
<td>Murine</td>
<td>MAPG × CD28 (melanoma-associated proteoglycan)</td>
<td>Protein L</td>
<td></td>
<td>Phase I/II: metastatic melanoma</td>
</tr>
<tr>
<td>MDX-H210 520C9 × H22</td>
<td>Medarex</td>
<td>Murine</td>
<td>HER2/neu × CD64</td>
<td>Fab chemical conjugate</td>
<td>Gel filtration chromatography</td>
<td>Phase I completed: 30 patients; advanced breast cancer</td>
</tr>
<tr>
<td>MDX-447 H245 × H22</td>
<td>Medarex</td>
<td>Fully human</td>
<td>EGFR × CD64</td>
<td>Fab chemical conjugate</td>
<td>Gel filtration chromatography</td>
<td>Phase I/II completed: 64 patients; head and neck cancer</td>
</tr>
</tbody>
</table>

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as others have led to disappointing results. In the latter case, different explanations can be put forward to explain the poor performance. For example, no consistent antitumor activity was observed in clinical trials with bispecific F(ab)2 chemical conjugate antibodies MDX-210 (HER2/neu × CD64), its humanized version MDX-H210 and MDX-447 (EGFR × CD64) [50, 70]. Retrospective analysis of preclinical studies suggested that this lack of efficacy may have been due to the fact that effector to target (E:T) ratios and concentrations of bsAb were too low [71]. The hybrid hybridoma-derived bsAb 2B1 (HER2/neu × CD16) was found to be toxic probably because of its Fc portion binding to other Fc receptors [72]. Another bispecific F(ab)2 antibody could not be fully evaluated in a phase I–II study because of an insufficient drug supply [64, 73]. Also, bsAbs triggering the TCR/CD3 complex have been shown to induce severe and unexpected side effects upon intravenous applications in humans [74, 75]. The mechanisms by which these side effects are generated are only partly understood [76].

rM28 is a bispecific tascFv directed to a melanoma-associated proteoglycan as well as the costimulatory CD28 molecule expressed on human T cells. This bsAb is currently in phase I/II clinical trial to analyze safety and efficiency of its intrallesional application with autologous PBMCs in patients with metastatic melanoma stage III/IV and unresectable metastasis (in total 14 patients are expected to be enrolled). This trial is conducted at the University Hospital of Tübingen in Germany. A nonexpected dimer form of rM28 (probably due to Db-like homodimerization) was found to bind to tumor target cells and induce a pronounced T cell activation in PBMC preparations without additional TCR/CD3 stimulation being required. This targeted supraagonistic stimulation of the CD28 molecule led to effective tumor cell killing after induction of NK cells [77, 78]. Importantly for in vivo application, most of the supra-agonistic activity of rM28 depends on its binding to melanoma target cells (T cells are activated only when the bsAb is bound to the tumor cell via its tumor antigen binding arm). It will be interesting to see if such constructs with tumor × CD28 specificity induce less severe side effects than bsAbs targeting the TCR/CD3 complex.

MT103 (also known as MEDI-538) is a single-chain bispecific recombinant antibody from Micromet’s BiTE product platform that binds both the CD19 antigen and CD3, for the potential treatment of CD19-positive B cell lymphoma. MT103 was shown to be effective in vitro against CD19-expressing B cell lines at concentrations as low as 10–100 pg/ml [79]. A chromium release assay indicated that cytotoxicity could be induced within 4 h with as little as 10 pg/ml of MT103, with E:T ratios as low as 2:1 and without prestimulation of T cells [80]. This is in contrast to other CD19 × CD3 bsAbs (quadroma-derived) [81] or Db formats [82] that not only required T cell costimulation by anti-CD28 antibody or preactivation of T cells using IL-2 but needed high E:T ratios and high concentrations of bsAbs. Multicenter phase I trials were initiated in 2002 investigating the safety and tolerability of a continuous infusion of MT103 over a 4- to 8-week period at escalating dose levels in patients with relapse-indolent NHL. Long-awaited preliminary clinical data of 19 patients were presented at the 11th Congress of the European Hematology Association in June 2006 [83]. In the first three patients groups (0.5 up to 5 mg/m²/24 h), no dose-limiting toxicities were observed. An evaluation of dose level 15 mg/m²/24 h is ongoing. Pharmacodynamic effects were observed at 5 and 15 mg/m²/24 h with complete depletion of malignant B cells as well as T cell expansion in the majority of patients. Three out of 5 patients receiving 15 mg/m²/24 h MT103 for at least 2 weeks showed clinical responses as assessed by central radiology. One patient had a complete tumor response and 2 patients showed partial tumor responses. These preliminary results provide the first clinical validation for MT103. Strikingly, MT103 shows clinical activity at a serum level that is significantly lower than that used in the clinic with the mAb Rituxan, a standard therapy for NHL [83].

In collaboration with Fresenius Biotech, TRION Pharma is developing intact bsAb, ‘Triomab®, which are produced by a quadroma cell line prepared by the fusion of two hybridoma cell lines (mouse IgG2a and rat IgG2b) [84]. This new class of antibodies can not only concomitantly recognize tumor cells (via binding to EpCAM or HER2/neu for catumaxomab and ertumaxomab, respectively) and T lymphocytes (via binding to CD3) with its two binding arms, but also activates Fcyl receptor-positive accessory cells through its Fc region (CD64+ accessory cells and CD16+ NK cells) [85]. In vitro, the Triomab-mediated killing of tumor cells results from cell-mediated killing (T cells, NK cells and FcγR-I+ cells) and a bystander effect via production of TNFα that induces apoptosis in target cells [86]. These bsAbs are considered as promising agents for the elimination of disseminated tumor cells.

To date, catumaxomab (EpCAM × CD3) has been tested in patients with malignant ascites caused by ovarian cancer [87], in patients with non-small-cell lung cancer and in patients with peritoneal carcinomatosis (PC)
secondary to gastrointestinal tract cancer. PC is a disease considered untreatable and the prognosis is very poor at 3–6 months. Recent results of a phase I/II trial enrolling 22 patients have demonstrated that the injection of catumaxomab into the abdominal cavity could be used to treat PC. The drug was delivered in 4 doses spread out over 10 days. The maximum tolerated dose was established to be 10 µg for a first infusion, and 200 µg as a fourth infusion. One patient had a complete response, and 3 others survived for up to 19 months posttreatment [88]. These observed clinical responses are encouraging as they indicate significant antitumor efficacy.

Similar results were observed with Triomab ertumaxomab targeting HER2/neu and CD3. The ability of ertumaxomab to kill HER2/neu-positive tumor cells was shown in various in vitro models. Furthermore, ertumaxomab was shown to completely eliminate autologous tumor cells in leukapheresis products of patients with breast carcinoma contaminated with tumor cells [89]. In a prospective study, 8 patients with malignant ascites were treated with an intraperitoneal (i.p.) application of ertumaxomab. The treatment showed convincing efficacy in patients with malignant ascites, with total elimination of tumor cells at doses as low as 40–140 µg. Moreover, the correlation between tumor cell elimination and long-term disappearance of ascites accumulation was shown [87]. Recently, 17 patients with metastatic breast cancer expressing HER2/neu were enrolled into a multicenter phase I dose-escalating trial [90]. Patients received up to three intravenous infusions of ertumaxomab. All patients developed symptoms of cytokine release syndrome. In general, the observed toxicities resemble the side effects seen after the administration of other therapeutic antibodies and were all fully reversible. One hundred micrograms were identified as the maximal tolerable single dose. Five out of 15 patients had an antitumor response 2 months after the last ertumaxomab infusion, one with complete response, two with partial responses and two with stable disease. With 25% of patients developing human anti-mouse antibody (HAMA) and 31% of patients developing human anti-rat antibody (HARA), the incidence of an immune response against mouse and rat antigens measured at 1 month after the last ertumaxomab infusion was rather low. Most clinical trials with intravenous application of other nonhumanized therapeutic antibodies in patients with solid tumors revealed HAMA/HARA rates of 40–80% following multiple exposures [91]. Furthermore, catumaxomab induced the development of HAMA/HARA in almost all tested patients (in 14 out of 15 patients and in all 8 patients with malignant ascites due to ovarian cancer and PC, respectively) after i.p. application. The low incidence of HAMA/HARA development observed in the study with ertumaxomab indicates a lower immunogenic potential of ertumaxomab after systemic intravenous application compared with the i.p. route. This observation is consistent with earlier investigations demonstrating that the i.p. and subcutaneous routes are superior to the intravenous route for inducing an immune response [92]. The significance of a relatively lower incidence of HAMA/HARA responses (31%) provides a rationale for the administration of a second treatment cycle in HAMA/HARA-negative patients. It remains to be seen whether lower HAMA/HARA responses are also observed in immunologically fully competent patients when ertumaxomab is given as an upfront treatment.

Conclusions

bsAbs enable novel therapeutic approaches such as re-targeting of toxic activities that are not possible with conventional mAbs. In addition, it has recently become more and more apparent that combination therapies with mAbs can be extremely beneficial in a number of disease settings. In this respect, bsAbs have a huge potential as dual targeting agents. They can accelerate the development of such therapies from a regulatory perspective by the simplified use of a single molecule instead of combination therapies. bsAb have therefore raised a lot of interest in the field of antibody engineering and this is reflected by the plethora of imaginative bsAb formats that have been developed as well as efforts in antibody purification strategies. The major challenge for these molecules to progress towards the clinic remains the large scale manufacturing of homogenous bsAb products. This has hampered the use of certain formats in early animal models but also led to the arrest of clinical trials because of product unavailability.

Nevertheless, very encouraging results have been obtained in clinical trials and in some cases strikingly low concentrations of bsAb were sufficient to show efficacy. This is of particular significance as a low dosage alleviates the pressure on manufacturing needs and drug supply. In addition, lower exposure of patients to bsAb potentially reduces the risks of immunogenicity. This might be an additional parameter to explain the low HAMA/HARA responses observed in patients after infusion of a mouse/rat bsAb in comparison to other nonhumanized therapeutic antibodies.
Most of the bsAbs that have reached the clinic so far were restricted to the field of oncology and derived from relatively early formats such as chemically cross-linked Fab fragments or nonhumanized antibodies isolated from quadromas. It is reasonable to expect that the benefit of using bsAbs will be explored in other therapeutic areas such as inflammation or infectious diseases. Finally, new bsAb formats are progressing towards the clinic; they will hopefully solve the remaining manufacturing issues so that the promises of these innovative molecules can be fulfilled.

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