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Advanced Drug Delivery Reviews 31 (1998) 105–129

advanced drug delivery reviews

# Bispecific antibodies for treatment of cancer in experimental animal models and man

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**Abstract**

Immunotherapy is a powerful anti-cancer treatment modality. However, despite numerous encouraging results obtained in pre-clinical studies, a definite breakthrough towards an established clinical treatment modality has as yet not occurred. Antibodies against tumor antigens have been shown to localise at the site of the tumor, but inadequate triggering of immune effector mechanisms have thwarted clinical efficacy thus far. Cellular immunotherapy has been hampered by limitations such as lack of specificity, down-regulation of major histocompatibility complex (MHC)-expression or Fas ligand up-regulation on tumor cells. This review focuses on the use of bispecific antibodies (BsAbs) for immunotherapy of cancer. Using BsAbs, it is possible to take advantage of the highly specific binding characteristics of antibodies and combine these with the powerful effector functions of cytotoxic immune effector cells. BsAbs share two different, monoclonal antibody-derived, antigen-recognizing moieties within one molecule. By dual binding, BsAbs reactive with a trigger molecule on an immune effector cell on the one hand and a surface antigen on a tumor target cell on the other are thus able to functionally focus the lytic activity of the immune effector cell towards the target cell. Over the last few years, the concept of BsAb-mediated tumor cell killing has been studied extensively both in preclinical models and in a number of phase I clinical trials. Promising pre-clinical results have been reported using tumor models in which diverse immune effector cell populations have been used. Despite this pre-clinical in vivo efficacy, the first clinical trials indicate that we are still not in a position to successfully treat human malignancies. This review discusses the production of BsAbs, the choice of trigger molecules in combination with potential effector cells and the preclinical models that have led to the current use of BsAbs in experimental clinical trials. It has become clear that appropriate immune cell activation and establishing a favourable effector-to-target cell ratio will have direct impact on the efficacy of the therapeutic approaches using BsAbs. New directions are discussed, i.e. finding appropriate dosage schemes by which immune effector cells become redirected without inducing hyporesponsiveness, defining possibilities for combining different immune effector cell populations and creating an in situ tumor environment that allows maximal tumoricidal activity 

*Keywords:* Bispecific antibodies; Immunotherapy; Cancer; Immune-effector-cell targeting; Cytolytic activity; Immune-effector-cell activation; T-cell; Macrophage; NK-cell

**Contents**



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0169-409X/98/$19.00 
*PII* S0169-409X(97)00096-3

## 1. Introduction

Over the years following the introduction of the hybridoma technology, a large array of monoclonal antibodies with predefined specificity for tumor-associated antigens (TAA) have been generated. In the beginning, these monoclonal antibodies (Mabs) evoked high expectations with regard to therapeutic and diagnostic efficacy [1,2]. Antibodies play a central role in humoral defence against non-self substances and can form a link with the cellular immune system by making opsonized targets susceptible to Fc-receptor (FcR)-positive cell-mediated phagocytosis and killing. For this reason, the application of Mabs directed against tumor antigens was regarded as a promising new type of immunotherapy. After various clinical trials exploring the potential of such tumor-specific Mabs, one had to conclude however that clinical application of Mabs as such is mostly ineffective. The main factors underlying the clinical inactivity of antibodies are: Incompatibility between the antibodies used and the effector functions (complement and/or antibody-dependent cellular cytotoxicity (ADCC) reactions) they are supposed to trigger, short in vivo half-life and immunogenicity of the antibody preparations. In addition, the few successes that have been documented thus far can not be attributed to a direct cytocidal effect of the applied antibodies, since responses are observed weeks to months after antibody treatment. Speculation on the actual effector mechanism underlying these cases included enhanced tumor cell processing, triggering a cellular anti-tumor response, or an anti-idiotypic cascade resulting in an ab2/ab3 antibody response as a result of the Mab treatment [3,4]. However, no proof for these mechanisms was found nor did it result in more effective Mab-based treatment protocols [4,5].

BsAbs may provide a tool to circumvent the above-mentioned drawbacks of monoclonal antibodies for therapeutic use [6]. BsAbs, with a chosen dual specificity for an activation molecule on an immune effector cell and a tumor associated antigen (TAA), allow simultaneous tumor cell binding and effector cell activation through crosslinking [7–9]. Since 1983, when Milstein and Cuello [6] first described the generation of BsAbs by the hybrid hybridoma technique, different BsAb-based retargeting approaches have been worked out, leading in 1990 to the first experimental clinical application of BsAbs [10]. Effective BsAb-mediated target cell killing has been shown in vitro for all immune cell populations with genuine killing potential and susceptibility of target cells to lysis seems to rely only on expression of the relevant target antigen [11–18]. The introduction of molecular biological techniques has given rise to new and more advanced bispecific antibody derivatives which, however, still have to prove their value.

## 2. Production of bispecific antibodies

### 2.1. Biological engineering of BsAbs

The biological preparation of BsAbs by hybrid hybridomas, or quadromas, is generally based on a method described by Milstein and Cuello [6]. Hybridoma cells producing Mabs of a desired specificity are modified to become sensitive to hypoxanthine-, aminopterin- and thymidine (HAT) containing medium using the drug 8-azaguanine and resistance to neomycin (neo), usually by retroviral infection. This results in a hybridoma cell line ($neo^r$/$HAT^s$) that is capable of growing in neomycin but not in HAT-containing medium. The second hybridoma cell line of interest, which will be HAT resistant/neomycin sensitive, capable of growth in HAT- but not neomycin-containing medium, is then selected ($neo^s$/$HAT^r$). After fusion of the two different hybridomas, the resulting quadroma cell line can be selected by culturing and cloning in HAT- and neomycin-containing medium. [19]. The hybrid hybridoma methodology is relatively simple and the resulting bispecific antibody is naturally assembled. A drawback of this technology is that quadromas can produce various, i.e. hypothetically up to ten, antibody species, formed by differential dimerization of the four heavy and light chains produced [2] (Fig. 1). Thus, the percentage of true BsAb produced by a quadroma cell line is rather unpredictable and laborious techniques are needed to separate the BsAb from the other antibody fractions. By careful selection of the original parental hybridomas, physical differences between the various antibody species can be introduced, which may be helpful in the process of BsAb purification.

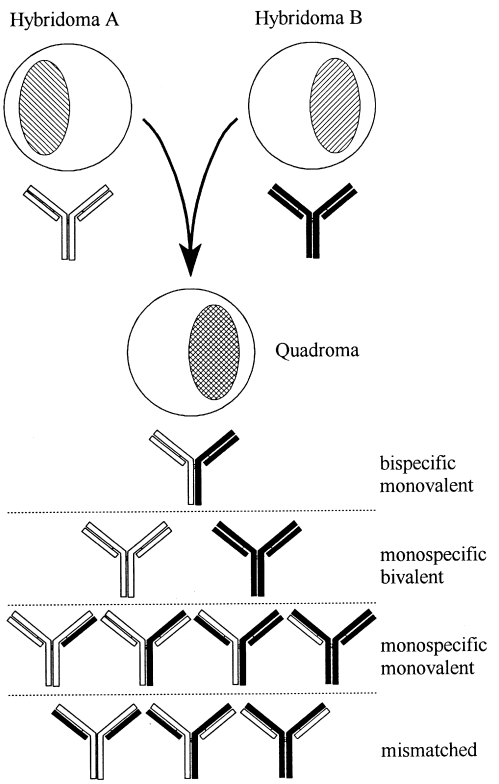


Fig. 1. Generation of bispecific antibodies by the hybrid hybridoma technology. Two hybridomas of the selected specificity are fused, resulting in the formation of a hybrid hybridoma or quadroma cell line. One clonal quadroma cell line is theoretically capable of producing up to ten different antibody species, which can be divided into four categories; monovalent bispecific, bivalent monospecific, monovalent monospecific (partially mismatched) and completely mismatched, based on the different heavy and light chain associations.

### 2.2. Chemical engineering of BsAbs

Antibodies derived from two different hybridomas can be coupled directly to each other using bifunctional cross-linking reagents such as N-succinimidyl 3-(2-pyridyldithiolpropionate (SPDP) [20,21]. However, cross-linking Ig molecules as such may result in a poorly defined and difficult to reproduce end-product. To overcome this, a more sophisticated approach has been described using 2-nitro benzoic acid (DTNB) or *o*-phenyl-dimaleimide (*o*-PDM), which are able to react with sulfhydryl groups present in the hinge-region of IgG molecules [12,22,23]. Purified IgG or enzymatically produced $F(ab')_2$ fragments thereof, are reduced under mild conditions yielding Fab′ reagents with free hinge-region SH groups that are covalently linked to DTNB or *o*-PDM. A second antibody, reduced to yield Fab′ fragments containing free hinge-region SH groups, are added and disulfide- or thioether-linked bispecific antibody molecules are generated by oxidation (Fig. 2). Both methods for the chemical assembly of two different antibody molecules have been shown to be suitable and yield relatively homogeneous bispecific antibody products. The *o*-

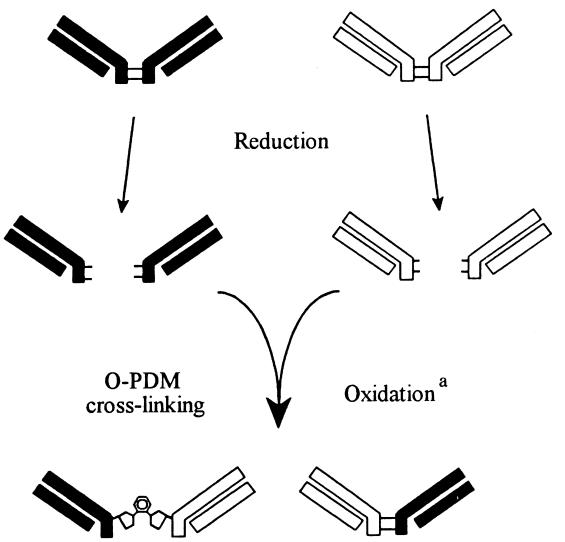


Fig. 2. Generation of bispecific antibodies by chemical cross-linking. Purified antibodies of a desired specificity are pepsin digested and mildly reduced to yield Fab′ fragments. Hinge-region SH groups are then saturated with bifunctional crosslinkers such as DTNB or *o*-PDM. A second antibody, reduced to yield Fab′ fragments containing free hinge-region SH groups, is added and disulfide- or thioether-linked bispecific antibody molecules are generated by oxidation. [a]Intrachain disulfide reoxidation and Ig homodimerization are prevented using arsenite and DNTB, respectively.

PDM linker has the additional advantage of providing the possibility for a third antibody fragment to be linked to BsAbs, yielding a trispecific antibody [12,24]. Still, heterogeneity of the end product may be expected due to incomplete enzymatic digestion, non-specific cleavage of the IgG chains during reduction and the formation of random interchain disulfide bonds during re-oxidation [25].

### *2.3. Genetic engineering of BsAbs*

The in vivo application of murine antibodies is limited by a number of characteristics. One problem that has been frequently encountered in clinical treatment is the immunogenicity of murine-derived Mabs [26]. Non-human amino acid sequences and glycosylations have been shown to be important factors rendering clinically relevant antibodies, such as CO17-1A, highly immunogenic to the human immune system, resulting in their neutralization by naturally occurring antibodies or the induction of strong human anti-mouse antibody (HAMA) responses [26,27]. Other typical problems relate to the relatively large size of the antibodies, which results in poor tumor penetration characteristics and incompatibility with human immune effector functions. Molecular engineering methods have been utilized to overcome these problems by manipulating the size, shape and affinity of the antibodies [28]. Humanized versions of murine Mabs have been designed where the variable chains of the original Mab are combined with the constant chains of human IgG to enhance effector functions and reduce the chance of a HAMA response occurring. Alternatively, the six hypervariable loops (CDRs) forming the antigen binding site of an antibody can be transplanted into a human framework, resulting in a CDR-grafted or humanized antibody. In addition to modified complete Ig molecules, recombinant DNA technology has been used to construct small antibody-like molecules called single chain Fv fragments (scFvs) [29–33]. ScFvs are recombinant antibody fragments consisting of only the variable light chain (VL) and variable heavy chain (VH) domains, covalently connected to each other by a flexible polypeptide linker. Due to their relatively small size (approximately 26 kDa), scFvs have rapid blood clearance pharmacokinetics and show an improved tumor penetration in vivo [34]. Until now, genetic engineering is conducted mostly on murine derived Mab sequences, isolated from hybridoma cell lines. Recently, systems have been described to produce completely human antibodies in vitro by phage display or in vivo using transgenic mice [35–37].

To date, various forms of genetically engineered BsAbs, some of which are depicted in Fig. 3, have been described. Firstly, scFv molecules, functionally expressed in *E. coli* have been modified to express a terminal cysteine by which they can be crosslinked using conventional sulfide-reactive crosslinkers [38]. Secondly, by interconnecting two different scFv constructs with a long enough flexible linker, so-called bispecific single chain Fv fragment [Bs(scFv)2] have been generated [39,40] (Fig. 3a). These latter molecules have the advantage that post-production modification, such as chemical coupling, is no longer needed. However, despite the inclusion of a signal sequence, $Bs(scFv)_2$ form aggregates

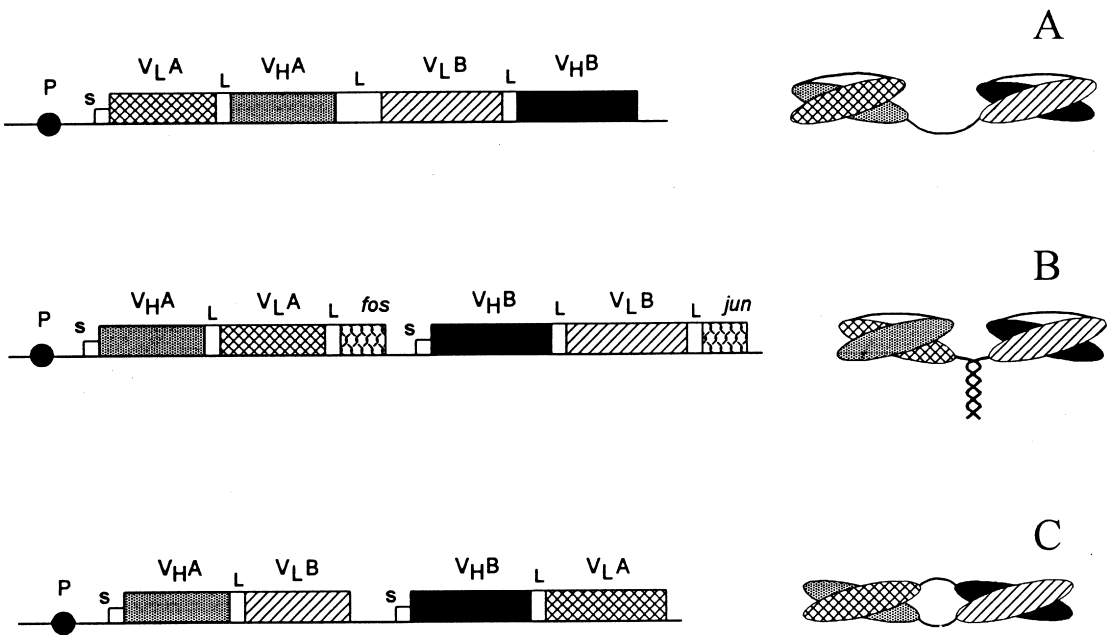


Fig. 3. Generation of bispecific antibody molecules by genetic engineering. (A) Genetic organization and protein assembly of bispecific $(scFv)_2$. (B) Genetic organization and protein assembly of fos–jun zippered bispecific scFv fragments. (C) Genetic organization and protein assembly of bispecific 'diabodies'. P, promoter region; s, signal sequence; VH, variable heavy domain; VL, variable light domain; L, linker. (Figure adapted from ref. [25] with permission).

within *E. coli* and their typical production yield is still too low for extensive experimentation or clinical application [41]. Thirdly, as an alternative to chemical crosslinking, scFv fragments can be coupled to form bispecific reagents using the fos–jun leucine zipper technique (Fig. 3b). This technique takes advantage of the property of the transcription factors fos and jun to spontaneously form stable fos–jun heterodimers [42]. Two different scFv fusion proteins are constructed, either containing fos or jun. Upon expression, fos–fos and jun–jun homodimers are formed, which can be monomerized by mild reduction. Subsequent mixing and oxidation of equimolar quantities of scFv–fos and scFv–jun monomeric fragments will preferentially yield bispecific fos–jun zippered antibody fragments with a typical recovery after purification and desalting of approximately 60%. To further minimize the size of these antibody constructs, pepsin digestion to remove part of the fos–jun zipper is possible [43]. Fourthly, in 1993, Holliger et al. [44] showed that when scFv molecules were constructed with no or very short linkers, intrachain pairing was no longer possible, but, instead, interchain pairing occurred. This phenomenon apparently resulted from a natural affinity of VH for VL and vice versa. This concept was exploited to develop so-called diabodies in which the VH- and VL-encoding regions of two different antibody specificities were expressed on two separate chains, each containing a linker short enough to prevent self assembly of the individual VH and VL domains (Fig. 3c). Simultaneous and equimolar expression of the two chains, e.g. using a dicistronic expression system, results in the formation of a bispecific molecule by dual interchain VH–VL pairing [45,46]. Optimizing the formation of true bispecific reagent has been described by introducing a disulfide bridge between one of the VH–VL chains. Although the percentage of bispecific diabody increased to 100%, the protein yield after expression dropped by about 100-fold [47].

In conclusion, the possibilities for genetic engineering of antibody fragments within the field of research of bispecific antibodies are numerous. The

next years will undoubtedly be marked by increasing knowledge of the possibilities for the production of high grade bispecific reagents in quantities that are sufficient to allow experimental and clinical testing.

## 3. Bispecific antibody triggered effector functions

### *3.1. Targets for BsAb-mediated cytotoxicity*

Many different TAAs have been described for targeted immunotherapy. General considerations that rationalize the choice of a target antigen are: (1) The expression of the antigen on tumor cells should be homogenous throughout the tumor and high enough to ensure effective binding of the antibody of choice. (2) Expression of the antigen by normal tissues should be limited or, if the antigen is expressed on normal tissue, it should be inaccessible to antibodies in these tissues. (3) The antigen should be membrane bound and not be shed from the cell surface. It should be realized that the heterogeneity of tumors as well as the fact that their antigenic make-up resemble the normal tissues from which they originate has made it difficult to identify suitable target molecules. In the following, a number of potential target antigens are discussed (see also, de Leij [48]).

The surface Ig idiotype sequences present in B-cell malignancies are close to ideal. However, anti-idiotype targeting has several drawbacks that are difficult to overcome. Firstly, the unique intrinsic specificity of the surface Ig implies that new antibodies have to be generated for every distinct malignant B-cell clone. Secondly, soluble malignant B-cell-produced antibody present in the serum may act as a scavenger for the therapeutic anti-idiotypic antibodies by preventing them from binding to their membrane bound target [49]. Other B-cell-specific target antigens include the normal B-cell markers CD19 and CD20, which are present on a wide range of B-cell-derived malignancies. Immunotherapy directed against normal B-cell-specific markers holds the risk of compromising the natural immune response by eradication of the complete B-cell repertoire. However, it may be anticipated that this immune-gap is being readily restored by new, bone-marrow-derived, B-cells. Recently, Link and Weiner [50] and Elsasser et al. [51] described an antibody that was reactive with an alternatively glycosylated HLA class II variant expressed by a significant number of B-cell malignancies. BsAbs derived from such antibodies considerably enhance the effector-to-target ratio and, hence, therapeutic effectiveness by selective BsAb-mediated recognition of malignant B-cells.

Examples of carcinoma-associated antigens that have been exploited in therapeutic protocols are c-erbB-1 or epidermal growth factor (EGF) receptor, c-erbB-2 or HER2/*neu* antigen, the folate receptor or folate binding protein (FBP) and epithelial glycoprotein-2 (EGP-2) [2]. Over-expression of the c-erB-1 proto-oncogene product was initially reported in 100% of squamous cell carcinomas of the lung [52], but later studies reported a 65 to 90% expression level [53]. Also, 15–40% of the adenocarcinomas and 25–40% of large cell carcinomas were found to over-express c-erbB-1 [54]. The proto-oncogene product c-erbB-2 is amplified in a variety of adenocarcinomas and squamous cell carcinomas, including lung, breast, gastric and colon cancer [55,56]. The antigen is also expressed in normal lung tissue as was shown by the presence of c-erbB-2 mRNA and reactivity with c-erbB-2 antibodies [56]. A number of both preclinical and clinical studies have used the folate receptor or FBP as a target for immunotherapy of ovarian carcinoma [57–61]. Expression of this tumor antigen by normal tissues is restricted [62]. The carcinoma-associated antigen, EGP-2, is a 38-kDa transmembrane glycoprotein, present on the majority of simple, stratified and transitional epithelia [63–67]. The biological function of EGP-2 has not yet been established. A role as growth factor binding protein has been proposed [68]. Others have suggested a role for EGP-2 as an epithelial-specific adhesion molecule. Indeed, Litvinov et al. [69] provided evidence that the EGP-2 molecule mediates a homophyllic intracellular interaction and might thus be involved in the organization and architecture of epithelial tissues.

### *3.2. Targeting toxic substances*

Immunotoxins have long been studied for their targeted destructive potential. Typical problems of this treatment modality are the chemical or genetic

modification of the toxin needed to decrease its affinity to normal tissue. In addition, the conjugation procedure of the toxin to the antibody seems critically important to allow, firstly, efficient internalization of the immunotoxin and, secondly, intracellular release of the toxin, which is needed to exert its toxic potential. A number of BsAbs have been constructed that allow the delivery of toxins either directly or in a two-phase concept. These BsAbs combine a specificity against the ribosome-inactivating proteins, saporin or ricin, with a specificity against cell surface receptors on tumor cells [70]. In yet another approach, pairs of BsAbs have been used against one toxin but different TAAs. Simultaneous ligation of the different TAAs resulted in an enhanced specificity of the immunotoxin for the target cells and an enhanced internalization of the immunotoxin complex by the target cells [71,72]. Similar results were obtained using pairs of BsAbs that ligate different epitopes on the toxin with the same cell surface receptor on the tumor cell [70,73,74]. Studies using BsAb-targeted toxin protocols for the treatment of lymphoma patients are currently under evaluation [75].

#### *3.2.1. The two-phase targeting model*

An interesting biphasic treatment strategy for the enhanced delivery of radionuclides to the site of the tumor was described by Goodwin et al. [76]. Preinjected BsAb, reactive with both a TAA and a chelator such as diethylenetriaminepentaacetic acid (DTPA), localises at the site of the tumor. Subsequently, after serum clearance of excess unbound BsAb, this is followed by injection of DTPA containing a radionuclide, which is then rapidly trapped at the site of the tumor by binding to the BsAb [77]. Using this treatment modality, different radioisotopes, such as $^{111}$In and $^{90}$Y, can be used for diagnosis and therapy, respectively. Also, a relatively new radioimmunotherapeutic modality, i.e. targeted boron neutron capture therapy [78], could very well benefit from this approach.

### *3.3. Targeting cellular effector functions*

A number of different immune effector cell populations have been studied extensively for potential BsAb-mediated killing capacity. These effector cell populations include T-cells (both CD4 and CD8) [12,13,15,16,79,80], natural killer (NK) cells [16,17,81], macrophages and polymorphonuclear (PMN) cells [11,18]. Only a selected set of surface receptors are able to initiate the lethal hit delivery program in the effector cell (Table 1). These include the CD3, CD2 and the T-cell receptor (TcR) on T-cells, CD2 and CD16 (Fc RIII) on NK and lymphokine activated killer (LAK) cells, and Fc RI, II, and III on monocytes, macro phages and PMNs. The amount of BsAb needed to endow effector cells in vitro with killing potential against tumor cells is minimal and lies in the microgram–picogram/ml range (nM–fM) [82,83].

#### *3.3.1. Myeloid effector cells*

BsAb-mediated target cell death induction by myeloid effector cells is triggered in a way that is analogous to the phenomenon of antibody-dependent cellular cytotoxicity (ADCC) in which antibodies bind to an antigen on a target cell via their variable region and to an Fc-receptor on a myeloid effector cells via their Fc-domain. Crosslinking of target cells with myeloid effector cells in this way induces cytolysis of the target cell [84]. Three types of FcR-γ can be found on myeloid cells: FcRI, (CD64), FcRII

Table 1
Triggering molecules for immune cell-mediated targeting

| Surface receptor | Cell type | Reference |
|---|---|---|
| TcR[a] | T-cells | [162] |
| TcR | T-cells | [208] |
| CD2 | T-/NK-cells | [208] |
| CD3 | T-cells | [20,83] |
| CD5[b] | T-cells | [9] |
| CD16 | NK-cells/LGL<br>PMN | [81,208,209] |
| CD28[c] | T-cells | [7–9] |
| CD32 | PMN/MQ<br>Macrophages | [210] |
| CD64 | PMN[d]/MQ<br>Macrophages | [84,210,211] |

TcR, T-cell receptor; LGL, large granular lymphocyte; PMN, polymorphonuclear leukocyte; MQ, macrophage.
[a]In a rat animal model.
[b]Effective when used in conjunction with CD28 and/or CD3.
[c]Effective when used in conjunction with CD2 or CD5 and/or CD3.
[d]When activated with IFN-γ or G-CSF.

(CD32) and FcRIII (CD16) [85]. These Fc-type receptors seem to be the only myeloid cell surface molecules that are able to induce activation of these immune cells and to trigger cytolytic capacity. The type I and III receptors appear to exploit a secondary signal transduction pathway that involves association with homodimers and chains which are also found in the TcR/CD3 complex in T-cells [86]. The type I receptor, which is constitutively present on macrophages and monocytes, seems to be most effective in the induction of target cell lysis. An approximately twenty-fold increment of expression of this receptor on neutrophils is possible by incubation with interferon-α (IFN-α) or granulocyte-colony stimulating factor (G-CSF) [84]. Since the FcR I has high binding affinity for monomeric IgG ($K_a$ $10^8$–$10^9$ $M^{-1}$), it is thought that this receptor is constantly occupied in vivo, presenting a problem in exploiting this receptor for in vivo ADCC activity. Bispecific antibodies directed against an epitope outside the IgG-binding site have been shown to circumvent this and to induce effective killing of target cells in the presence of physiological concentrations of human IgG [87]. One of these BsAbs, the chemically engineered MDX-210, has been shown to induce efficient lysis of HER-2/*neu*-positive target cells by monocytes and G-CSF-stimulated neutrophils [10,88]. This BsAb is currently being evaluated in clinical phase I–IIa trials.

Besides direct bispecific antibody-induced cytotoxicity, additional immune effector mechanisms may be triggered through enhanced antigen presentation [89,90]. Specific, BsAb-mediated, tumor cell phagocytosis by myeloid effector cells might accomplish processing of the tumor cells by so-called 'professional antigen presenting cells' (APC), which brings about a BsAb-independent antibody or cytotoxic T-lymphocyte (CTL)-driven immune effector response.

### *3.3.2. NK-cells*

NK cells are cytotoxic lymphocytes with a ''natural'', MHC-unrestricted killing potential. A variety of tumor target cells are susceptible to NK cell-mediated lysis. Cytolytic potential and the range of potential targets can be increased considerably by culturing NK cells in interleukin-2 (IL-2) or IL-12 [91–94]. The receptors on NK cells involved in target cell recognition have not been fully characterized [95,96]. Target cell recognition can be induced also by target cell-specific antibodies, which can activate NK cells through binding via their Fc-domain to the FcRIII (CD16), which is constitutively expressed by NK cells [84]. The FcRIII is linked to a signal transduction pathway, leading, upon antibody-mediated NK–target cell interaction, to NK activation and enhanced cytolytic potential, similar to the potentiating activity of exogenously applied IL-2 [92,93]. BsAbs directed against CD16 and a TAA have been shown to be able to efficiently direct the lytic potential of NK cells towards TAA-positive target cells [16,81,97]. Additionally applied IL-2 and IL-12 seem helpful in further increasing the activity of NK cells and BsAb-mediated target cell killing, but might also be dispensable [94,95,98]. 2B1, a BsAb produced by a hybrid hybridoma, combines specificity for the FcRIII (CD16) present on neutrophils, macrophages and NK cells, with specificity for the HER-2/*neu* oncogene product [99]. This BsAb is currently being evaluated in a phase I/II clinical trial [99].

### *3.3.3. Cytotoxic T-cells*

In contrast to NK- and myeloid effector cells, CTLs need prior activation in order to gain cytolytic potential [100–102]. Normally, T-cells engage their targets via interaction of the TcR with peptide/MHC molecules. This results in TcR crosslinking followed by signal transduction (Fig. 4a). Besides TcR-mediated triggering, additional co-stimulatory signals are needed for a T-cell to become fully active and cytotoxic [103]. In this respect, it is well known that the CD28/B7 interaction has an important role in enhancing IL-2 production and up-regulation of the high affinity IL-2 receptor chain [104–106]. It is most likely that a complex network of interactions is required for T-cells to become maximally active. Besides CD28, cellular interactions involving CD2, LFA-1, ICAM-1, CD5, CD40 and, in addition, the presence of various cytokines, such as IL-2 and tumour necrosis factor (TNF), play a role in the additional activation of T-cells [107–111]. Once activated, T-cells presumably form the most potent cytocidal effector cells, being able to kill multiple targets in sequence [112]. The strict MHC restriction that applies to both activation and target cell killing

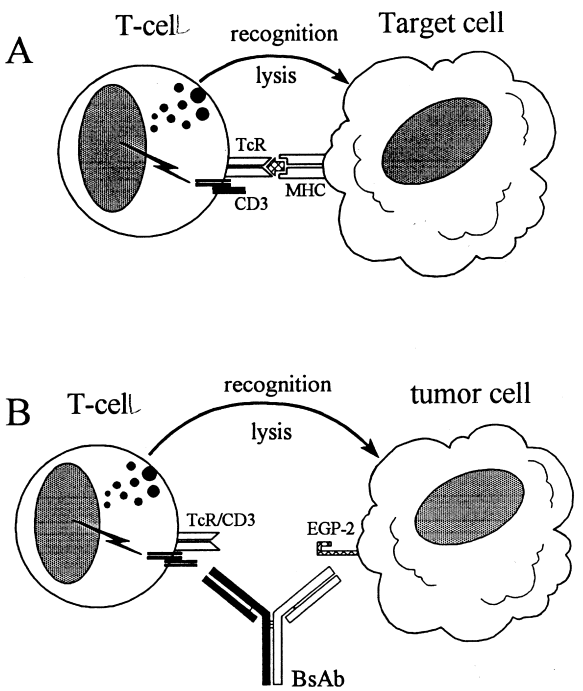


Fig. 4. Killing of target cells through (A) target cell-specific CTL, recognizing specific peptides presented by MHC class I molecules at the surface of the target cell via the TcR expressed by the CTL. (B) Bispecific antibody redirected CTL recognizing any bispecific antibody-defined target antigen-positive target cell. TcR–MHC- or BsAb-mediated interaction between the CTL and the target cell initiates CTL activation by which the cytolytic activity of the CTL is directed towards the recognized target cell, which is subsequently killed.

imposes, however, practical problems with respect to the use of T-cells for the eradication of a target. Bispecific antibodies have proven to be able to circumvent this MHC restriction. By dual binding to both an invariable determinant of the TcR/CD3 complex and a target cell surface marker, the BsAb specifically directs the lytic potential of the CTL towards the target cell as though a specific TcR–MHC interaction had been initiated [83,113] (Fig. 4b). Especially in the light of the need for costimulatory signals involved in T-cell activation, as indicated above, the use of T-cells as immune effector cells for BsAb-based treatment concepts is somewhat more complex when compared to NK- and myeloid effector cells. Combinations of BsAbs triggering various stimulatory and costimulatory molecules on T-cells seem appropriate to efficiently induce effective T-cell activation [7–9,114]. T-cells form a population of immune effector cells that may be naturally involved in the management of cancer [115,116]. Naturally occurring tumor regressions are always characterized by a marked infiltration of T-cells [117] and, thus, when exploited properly, T-cells may be considered to represent an effector cell population with effective anti-tumor activity.

## 4. Preclinical application of bispecific antibodies

### *4.1. In vitro characteristics of BsAb-mediated CTL-induced cytotoxicity*

Not only direct cytolytic activity resulting in acute target cell death is observed upon BsAb-induced effector–target interaction. During CTL-based cytotoxicity, in which both CD4- and CD8-positive cells have been implicated, cytokines, which seem to be essential for the activation status and function of the effector cells, are released [118]. These cytokines are not only beneficial to the activity of the effector cells but seem to induce growth inhibition of target cells as well [119,120]. Therefore, the efficacy of in vivo treatment is not solely based on the direct delivery of a lethal hit but also on a more indirect growth inhibitory effect. The latter phenomenon is characterized by the release of multiple cytokines such as IFN-γ and TNF-α [121], which participate in both a tumor cell cycle arrest [120,122], up-regulation of TAA expression and activation of immune effector cells [123].

Direct BsAb-mediated cytolysis of target cells is characterized by two distinct forms of cell death [124,125]. Firstly, necrosis occurs. This process is defined by a loss of integrity of the cell membrane and total disintegration of the cellular constituents. This form of target cell death is easily assessable in vitro, e.g. by the release of pre-incorporated labels such as $^{51}$Cr. Secondly, apoptosis is observed. This form of target cell death is characterized by morphological changes, such as target cell membrane blebbing, nuclear condensation followed by DNA fragmentation and the formation of apoptotic bodies [124]. Being more difficult to quantify, this form of cell death is not often assessed. However, it may be the more relevant way by which target cells are

eliminated in vivo. In this respect, it should be kept in mind that effective target cell lysis in vitro often relies on apparently non-physiological effector-to-target ratios. A physiologically relevant in vitro cytotoxicity assay was described by Haagen et al. [126] in which a five log kill of tumor cells was attained at an effector-to-target ratio of 3:1. In this assay, cytotoxicity was assessed in a long term clonogenic assay. Repeated dosing of BsAbs and exogenous IL-2 was needed to maintain the killing capacity of the CTL. The observation that repeated dosing of BsAbs is required to consolidate the killing capacity of CTL is in line with earlier data published by Blank-Voorthuis et al. [127]. They showed that TcR molecules, engaged in the delivery of a cytolytic hit, were refractory to subsequent signalling. However, the cytolytic activity of these CTLs could be restored by adding fresh BsAb. Whether this represents a specific BsAb-related phenomenon or is inherent to CTL activity in general is not known. Despite the similarities between genuine and BsAb-mediated CTL triggering, there are important differences. BsAb–mediated T-cell triggering occurs via high affinity interactions mediated via Mabs, whereas genuine CTL activation relies on the physiological low affinity interaction established via the TcR–peptide/MHC complex [128–130]. The impact of this on T-cell triggering is of importance, as will be discussed below.

#### *4.1.1. Effector cell activation*

The importance of immune activation for the induction of anti-tumor activity by immune effector cells has been demonstrated in many studies. In vitro-activated peripheral blood mononuclear cells (PBMCs) show considerably higher lytic activity towards tumor cells when compared to resting PBMCs [100,102,131–133]. Furthermore, effector cell activation is likely to enhance their migration into tissues, which is an important prerequisite for the induction of effective anti-tumor activity in vivo. The in vitro activation and expansion of cytolytic T-lymphocytes is relatively simple and can be achieved by using Mabs directed against the T-cell receptor/CD3 complex that is present on all T-lymphocytes, in combination with exogenously added low doses of IL-2 [100,133,134]. Also, lectins, such as phyto haemaglutinin (PHA), or Mabs against other triggering molecules present on T-lymphocytes, such as CD2 in combination with CD28, have been successfully used to generate a CTL population exerting high lytic potential [7,110,135]. The prerequisites for the in vivo activation of CTLs are much less clear. Induction of T-cell activation in mice, using a CD3-recognizing Mab was successful [136–138], but clinical trials initiated to achieve CTL activation in humans using the CD3-recognizing Mab, OKT3, were unsuccessful [139–143]. In line with this, it was found that so-called 'whole blood cultures' (heparinized blood diluted 1:10 in RPMI 1640) performed in vitro show a poor proliferative response to added CD3 Mab, whereas isolated PBMCs were highly sensitive to these Mabs [144]. Studies have indicated the importance of prior or co-activation of CTL also for the induction of BsAb-mediated anti-tumor activity in vivo. IL-2, the superantigen staphylococcal enteroxin B (SEB), β-glucan or additional CD28-mediated costimulation have been shown to induce immune activation, which considerably enhanced the BsAb-directed cytolytic anti-tumor response in vivo [49,114,145–147]. BsAb-mediated in vitro activation of CTLs has been described through simultaneous crosslinking of selected CTL cell surface receptors with target cells or, alternatively, FcR-positive cells [7,145,148]. If and how these data can be extrapolated to the in vivo situation is not entirely clear yet. It has been shown that T-cell activation occurs in vivo, using an Fc domain containing anti-CD3 anti-TAA BsAb, both in the presence and absence of relevant tumor cells, whereas $F(ab')_2$ fragments from the same BsAb failed in this respect, even when applied at higher or repeated doses [145]. In contrast, using a combination of exogenously applied IL-2 and BsAb $F(ab')_2$ fragments, functional T-cell activation was induced and was shown to be confined to the presence of relevant target cells. In another approach, BsAbs directed against CD2 and the TcR of the rat were shown to induce clear T-cell activation, which appeared to be confined to the CD8-positive cells (Dr. M. Glennie, UK, pers. commun.). Furthermore, severe toxicity was observed in a rat model upon i.v. administration of intact anti-TcR or related BsAbs, which could be attributed to an Fc-mediated immune activation [122]. Taken together, CTL activation using IL-2 or SEB in combination with BsAb $F(ab')_2$

fragments seems more appropriate for inducing immune activation and directed anti-tumor activity when compared to complete BsAb Ig. Specific and effective T-cell activation can be induced using anti-CD3 × anti-tumor BsAb in combination with BsAb directed against tumor antigens and costimulatory molecules on T-cells, such as CD28 and/or CD5 [7–9,114]. Such local activation strategies might abolish the need for continuous additional immune activation, as provided by, for example, exogenous IL-2, and thus limit systemic toxic side effects. In addition, activation strategies based on the triggering of combinations of stimulatory and costimulatory molecules might more closely mimic a natural T-cell response. This may initiate immune activation resulting in the establishment of a specific anti-tumor CTL response, including the development of a CTL memory population that provides protection against subsequent tumor challenges [149].

The CD3 complex is a very effective T-cell surface marker to trigger the lytic capacity of T-cells. However, during anti-CD3-mediated activation, either via interaction with the peptide/MHC complex, Mab or BsAb, TcR/CD3 down modulation is induced, resulting in a state of T-cell hyporesponsiveness to subsequent CD3-mediated triggering [49,128,129,150]. This might have adverse effects on the subsequent killing capacity of the CTLs and should be kept in mind when performing clinical phase I dose escalation trials, in order to define a maximal tolerable dose (MTD) and to study BsAb-related toxicity. Using saturating amounts of CD3-based BsAbs, rapid down-regulation of the TcR–CD3 complex is induced [150], which might subsequently result in decreased effector functions. Indeed, toxic side effects, such as those induced by TNF-α, seem to subdue after repeated BsAb application [89,151,152]. Different approaches might be followed to prevent this induction of hypo-activity. Alternative triggering pathways to induce T-cell activation may be used. In particular, the combination of CD2 and CD28 [110,153] might be useful in this respect, but CD5 together with CD28 can also significantly activate T-cells in the absence of triggering via CD3 [9,154,155]. One of the roles of costimulation in vivo during natural immune activation is to lower the threshold for T-cells to become activated through specific TcR–MHC interaction [128,156,157]. In this way, unnecessary down-modulation of the TcR complex is avoided and the activation of T-cells is tuned towards places that provide costimulatory help, such as in lymphoid tissues. Following this line of argument, lower doses of anti-CD3 × anti-tumor BsAb, applied in concert with other antibodies against costimulatory receptors, such as CD28 and CD5, could be used to induce effective T-cell stimulation. This would prevent CD3 modulation, i.e. not rendering the T-cells unresponsive to new doses of the CD3-based BsAbs. In particular, by simultaneously triggering CD3, CD5 and CD28 at the cell surface of CTL, efficient and rapid CTL activation can be induced [9].

### *4.2. Animal models*

The development of a therapeutic concept dealing with intervention in a complex and balanced network such as the immune system needs careful experimental foundation. This can only be achieved in immunocompetent in vivo models that reflect the clinical situation as close as possible. A number of animal models have been described to assess the characteristics of BsAb-mediated anti-tumor activity in vivo. In 1987, Titus et al. [158] showed anti-tumor activity in nude mice when they injected human tumor and effector cells together in the presence of BsAbs. The first immunocompetent models were described in 1991. Two groups independently described the efficacy of BsAb-mediated anti-tumor activity using the same B-cell lymphoma in a syngeneic mouse model [159,160]. A single intravenous injection of 5–10 μg of BsAb proved to be sufficient for prolonged survival of mice that had received a tumor inoculation two days prior to the treatment. However, increasing the tumor burden considerably reduced the therapeutic effectiveness. Restoration of anti-tumor effectiveness was possible by in vivo allosensitization of the T-cells [159]. Thus, the concept of BsAb-mediated tumor cell killing was shown to be successful with two parameters being critical, i.e. a low tumor burden and additional immune activation besides the presence of relevant BsAbs. There are several ways to induce additional immune activation [114,146,161–163]. However, immune activation in vivo is a complex process, with potential risk of escalation resulting in

high systemic toxicity. Beun et al. showed that, depending on the BsAb isotype used, FcR-positive cells, such as monocytes and macrophages, can crosslink a BsAb and mediate in the (unwanted) activation of T-cells [122]. Such T-cell activation is accompanied by severe toxic side effects that are reminiscent of the phenomena seen in human transplant recipients treated with high doses of the anti-CD3 Mab, OKT-3 [164,165]. Thus, considering the fact that tumor cells will not be directly accessible from the circulation, it seems preferable to use $F(ab')_2$ fragments of the BsAb in combination with additional immune activation signals, such as IL-2, providing activation and migration of BsAb-targeted T-cells. Such protocols may prevent CD3-mediated systemic T-cell activation through crosslinking via FcR-positive cells. Following this line of argument, a number of additional activation stimuli have been used in concert with BsAbs. These include antibodies to costimulatory molecules, cytokines, superantigens and lectins, all of which were proven to provide both necessary and effective costimulatory signals [88,114,146,147,162].

The role of tumor load and the dose of BsAb administered has been addressed in a number of studies published by Demanet et al. [49,159,166]. It had been shown that multiple doses of BsAb enhanced or restored the killing capacity of CTLs in vitro [126,127]. However, multiple BsAb injections did not result in increasing a favourable in vivo response when trying to eradicate higher tumor loads in the 38C13 lymphoma model [49]. BsAbs induced CTL hyporesponsiveness in a dose-dependent manner, in a way analogous to the results reported recently by Valitutti et al. [129] and Viola and Lanzavecchia [128] was found to impede effective CTL function. Dose–effectiveness studies showed an optimal biological dose by which increasing tumor loads could be eradicated successfully [49]. Furthermore, costimulation via CD28 was shown to enhance the therapeutic effectiveness by reducing the concentration of BsAb needed to successfully trigger CTL function, results which are in line with those of Viola and Lanzavecchia [128]. Similar results have been reported by Renner et al. [114], who showed the need for costimulation via CD28 to successfully eradicate an established Hodgkin's tumor in a SCID mouse model reconstituted with prestimulated human peripheral blood lymphocytes (PBLs). Selective infiltration of CTLs in the tumor was noticed in animals treated with BsAbs. No significant difference was found with respect to CTL infiltration between animals treated with CD3 + CD28-based BsAbs and animals treated with CD3-based BsAb alone. Apparently, in situ CTL activation dictated the favourable effectiveness of the CD3 + CD28-based BsAb treatment. Both in the model of Demanet et al. and the model of Renner et al. [114], priming or T-cell preactivation seemed necessary to elicit an effective anti-tumor response, suggesting that other interactions besides CD28 might be crucially important.

Until now, studies have focused primarily on the efficacy of immunotherapeutic modalities. Frequently and rather unexpectedly, high toxicities are encountered in patients treated with immunomodulatory agents [167]. It is unclear whether this toxicity results from specific or non-specific immune activation and is beneficial or not for overall anti-tumor effectiveness. It would be interesting, therefore, to put effort into the development of animal models that can be used to investigate treatment-related toxicity besides or in relation to effectiveness.

## 5. Clinical application of bispecific antibodies

Experience with BsAbs in clinical treatment settings is summarized in Table 2. Initial clinical experience with BsAb-targeted cellular immunotherapy was gained with autologous ex vivo activated PBLs. These were infused locally, e.g. intracranially [14], intraperitoneally [61,168] or intrapleurally [168], in the presence of BsAbs directed against the TcR/CD3 complex and a TAA. Induction of cytolytic anti-tumor activity was reported in all studies. Furthermore, an inflammatory reaction was induced, with elevated local levels of TNF-α and IL-6, a systemic acute phase response, and a local influx of polymorphonuclear cells at the site of injection. Abdominal fibrosis was observed in patients receiving intraperitoneal treatment [61,168,169]. Since the clinical relevance of locally applied BsAbs is limited, a number of studies were initiated for combined i.p./i.v. or just i.v. treatments of cancer patients with BsAbs. In 1992, the first

Table 2
Clinical application of bispecific antibodies (BsAbs)

| Cancer type | BsAb | | Application | Effector cell type | Reference |
|---|---|---|---|---|---|
| | Specificity | Format | | | |
| Glioma | NCAM × CD3 | IgG | i.c. | CTL[a] | [14] |
| Carcinoma | EGP-2 × CD3 | IgG | i.p./i.pl. | CTL[a] | [168] |
| Carcinoma | FBP × CD3 | IgG | i.p. | CTL[a] | [61,169] |
| Carcinoma | EGP-2 × CD3 | F(ab′)2 | i.v. + sc IL-2 | CTL | [167] |
| Carcinoma | HER/2 × FcRI | F(ab′)2[b] | i.v. | monocytes/MQ | [90,99] |
| Carcinoma | HER/2 × FcRIII | F(ab′)2[b] | i.v. + i.v. G-CSF | PMN | [171] |
| NH-lymphoma | CD19 × CD3 | IgG | i.v. | CTL | [152] |
| NH-Lymphoma | CD22 × saporin | F(ab′)2[b] | i.v. | CTL | [205] |
| Carcinoma | CEA × DTPA | F(ab′)2[b] | i.v. | CTL | [206,207] |

NCAM, neural cell adhesion molecule; EGP-2, epithelial glycoprotein-2; FBP, folate binding protein; DTPA, diethylene triamine pentaacetic acid; i.c., intra-cranial; i.p., intra-peritoneal; i.pl., intra-pleural; i.v., intra-venous; sc, subcutaneous; CTL, cytotoxic T-lymphocytes; MQ, macrophages; PMN, polymorphonuclear leukocytes.; NH, Non-Hodgkin.
[a]Ex vivo activated autologous CTL.
[b]Chemically constructed.

clinical pilot study was performed with intravenously applied BsAbs reacting with Fc RI and CD15 in four patients with CD15 + tumors [170]. No toxic side effects from the treatment were reported. More recently, a number of clinical trials have been described in which BsAbs were applied i.v. to patients with renal cell carcinoma (RCC) [167], Her-2/*neu* + breast and ovarian carcinoma patients [90,99,171] and CD19 + lymphoma patients [152]. Since activation and migration of T-cells is a prerequisite for effective BsAb-mediated cellular cytotoxicity, some of these studies were conducted in the presence of additional immune activation, such as that provided by IL-2 or G-CSF [167,171]. No or minor anti-tumor responses were observed in the patients treated in these i.v. phase I protocols thus far. However, some interesting results, suggestive of immune activation, possibly at the site of the tumor, have been described and will be given below. As discussed above, a particular problem that has to be dealt with in these phase I trials is the fact that the maximal tolerable dose does not necessarily correlate with the optimal biological dose. Dosing, timing and combination of the application of BsAbs with other, adjuvant immune stimulatory signals, such as exogenously applied IL-2, anti-CD28 Mab or anti-CD28-based BsAbs, will have to be further investigated.

### *5.1. Toxicity*

Toxicity, as seen after local BsAb application, is generally mild or absent [14,61,168]. Also, simultaneous i.p. and i.v. application of BsAbs redirected ex vivo activated T-cells did not induce serious dose limiting toxicity [169]. In contrast, direct intravenous application of BsAb can be associated with significant toxicity, which seems to be partly dose-related and most likely results from the release of inflammatory cytokines, such as TNF-α, IL-1, IL-6 and IFN-γ [60,90,152,167,171]. Using diverse BsAbs, targeting different immune effector cells, cytokine release profiles described in the various studies thus far do not seem to be fundamentally different. TNF-α is likely to play a central role in the observed toxicities and is released as early as 1 to 1.5 h after the start of BsAb infusion [152,167] (Fig. 5). IFN-γ is found in studies using targeted T-cells [60,167] but was not observed in a CD3 × CD19 or NK–effector cell-based treatment setting [99,152]. In addition, IL-6 and IL-8 levels significantly increased following infusion of FcRIII-based BsAbs [99]. Using myeloid effector cells, targeted via the FcRI, TNF, IL-6 and, especially, G-CSF were significantly released, whereas IFN-γ did not seem to be produced [90,171]. The relation between the BsAb dose administered and the concurrent toxicity is not always

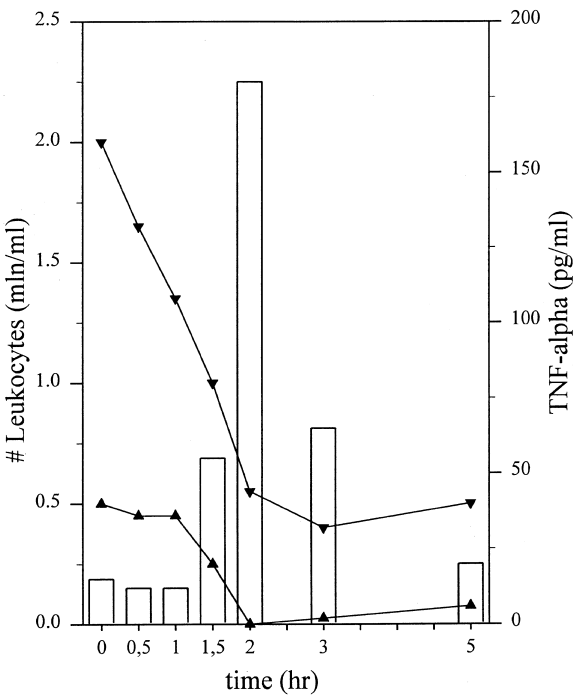


Fig. 5. Changes in absolute numbers of lymphocytes (d) and monocytes () from a patient during and after infusion with the bispecific antibody BIS-1 $F(ab')_2$ (CD3 × EGP-2) at a dose of 5 μg/kg, together with plasma levels of TNF-α (bars). (Figure reproduced from ref. [172] with permission).

clear. No toxicity has been described using very low BsAb doses, i.e. 100 μg. At higher doses, ranging from 0.2 to 18 mg of BsAb per infusion, highly variable toxicity has been noticed. Toxicity included flu-like symptoms, rash, chills, dyspnea, nausea and vomiting [90,167]. An interesting phenomenon that has been described is the reduced severity of the toxicity upon repeated intravenous application of BsAbs [90,151,152]. It would be interesting to know if this observed reduced toxicity can be explained by the above-discussed modulation of triggering receptors at the surface of immune effector cells. If so, the reduced toxicity might mirror a decreased in vivo effectiveness of the redirected immune cells. If the cytokines responsible for the toxicity are functionally needed in the process of anti-tumor activity, as has been described [119,122], the toxicity could be indicative of BsAb-mediated anti-tumor immune effector functioning. Intervention to reduce toxicity could, in that case, directly or indirectly reduce the efficacy of a BsAb-mediated anti-tumor response. Due to the phenomenon of immune effector cell desensitization, as discussed above, it has been difficult to define an MTD for BsAbs. Using $F(ab')_2$ fragments of a CD3 × EGP-2-reactive BsAb, BIS-1, we initially reported an MTD of 5 μg/kg in RCC patients receiving sc IL-2 in addition to the BsAb applied i.v. [167]. However, the occurrence and severity of toxicity is inconsistent and this MTD is currently being re-evaluated in an extended phase I trial in which patients are treated with multiple doses of up to 13 μg/kg [151]. In another phase I trial in which T-cells were targeted towards lymphoma using a CD3 × CD19 reactive BsAb, SHR-1, no MTD could be defined in doses of up to 5 mg/patient [152]. Using NK effector cells targeted against adenocarcinomas, using the BsAb 2B1, reactive with the FcRI and the HER-2/*neu* antigen, Weiner et al. [99] reported an MTD of 2.5 $mg/m^2$, which was defined by the occurrence of dose-limiting thrombocytopenia. No MTD was established using a BsAb, MDX-210, targeting FcRIII-positive myeloid effector cells against HER-2/*neu*-positive ovarian and breast cancer [90]. In the past few years, a number of groups have set out to define the feasibility of BsAbs applied i.v. in phase I trials. Toxicity seems to be an all or nothing phenomenon, which occurs above a difficult to identify dose of BsAb. Complicating factors in this respect are concurrently applied cytokines, which will most likely be needed to attain full cytolytic anti-tumor activity and the accessibility of the tumor for the redirected immune effector cells. From the foregoing discussion, it seems clear that it will be very difficult, if not practically impossible, to define a MTD as a basis for further BsAb-based phase II/III trials. Moreover, overshooting one's goal may occur if the optimal biological dose proves to be independently defined with no relation to the MTD [49].

### *5.2. Immunobiological aspects*

The most prominent and acute clinical manifestation of biological activity of applied BsAbs is the release of cytokines. In a phase I treatment protocol for RCC with a BsAb, BIS-1 $F(ab')_2$, during concomitantly applied IL-2 (sc), we observed a rapid transient cytopenia of lymphocytes, mono-

cytes and eosinophils in which CD11a brightly positive cytotoxic T-cells were found to leave the circulation preferentially [167,172]. Although monocyte depletion from the circulation was most impressive, lymphocytes started to leave the circulation first, i.e. almost directly after the start of the BsAb infusion (Fig. 5). It is tempting to speculate that BIS-1 $F(ab')_2$-mediated crosslinking of T-cells with EGP-2 positive tumor cells occurred, resulting in the release of cytokines, which then induced the cytopenia. Interestingly, neutrophils did not leave the circulation, suggesting an important role for VLA-4 in the marginalization or extravasation of the other leukocytes [150]. In contrast to this, targeting NK- and myeloid effector cells via an FcRIII × HER-2/*neu* and FcRI × HER-2/*neu*-reactive BsAb, 2B1 and MDX210, Weiner et al. [99] and Repp et al. [171] reported a transient grade 3 to 4 neutropenia. Similar results have been described by others [90,152]. The leukopenia, which can be almost absolute for certain cell types such as monocytes, has an onset at about 1 to 1.5 h after the start of the BsAb infusion and is most prominent at 2–3 h [150,167,172] (Fig. 5). Over the next 6–24 h, the leukocyte numbers return to normal levels again. The peak of leukocyte disappearance from the blood coincides with peak levels of TNF-α released in the blood. The appearance of other cytokines, such as IFN-γ, IL-6 and IL-8, is somewhat delayed, compared to this first release of TNF-α [60,152,167].

The way by which leukocyte migration into tumor tissue can occur has been the subject of many studies and is certainly of major importance in the optimization of BsAb-mediated cellular immunotherapy. At sites of inflammation, enhanced lymphocyte emigration will occur, due to up-regulation of adhesion molecules on endothelial cells and as a result of the local presence of elevated levels of chemokines [173,174]. In general, selectins as well as inter cellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) are readily up-regulated by inflammatory cytokines such as TNF-α and IFN-γ [168,175,176]. Furthermore, IL-2 itself might directly or indirectly promote leukocyte transmigration [177–180]. It is still unclear whether intrinsic differences between normal and tumor-associated endothelium are such that leukocyte extravasation is promoted or inhibited at tumor sites. Also, is it unclear how inflammatory cytokines, such as TNF-α and IFN-γ may influence these characteristics [174,178,181,182]. Renard et al. [183] reported significant up-regulation of adhesion molecules on tumor endothelium in biopsies taken from patients treated with high doses of TNF-α in an isolated limb perfusion setting. The up-regulation of adhesion molecules was associated with extensive leukocyte migration into the tumor in which polymorphonuclear cells preceded the development of a lymphocytic infiltrate. More recently, these observations have been challenged by Griffioen et al. [184] who observed down-regulation of adhesion molecules and decreased responsiveness of isolated tumor endothelium to inflammatory cytokines. Tumor-derived angiogenic growth factors, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), were shown to have different effects on immune effector cell adhesion to tumor endothelium and might thus have an important impact on the effectiveness of immune cell-based treatment protocols [182]. We have recently observed increased in vitro adherence of lymphocytes to resting endothelium, after preincubation of the lymphocytes with the anti-CD3 × anti-EGP-2 $F(ab')_2$ BsAb, BIS-1 (manuscript in preparation). If this also occurs in vivo, it might explain the rapid but transient leukocyte marginalisation observed in patients upon BsAb infusion. Despite this potential problem, it is clear that evoking a controlled tumor site-directed inflammatory reaction, by which immune effector cells become activated locally at the site of the tumor, could have a favourable impact on the effectiveness of BsAb-based cellular anti-tumor treatment [185–187].

Effective in vivo anti-tumor activity has been assessed most directly in local treatment protocols that allow post-treatment evaluation of the site of treatment [61,168,169]. Local, i.p. and intrapleural application of the BsAb BIS-1 together with ex vivo activated autologous lymphocytes resulted in a local anti-tumor response, as shown by immunocytological evaluation of intraperitoneal samples taken after treatment and also by decreased local carcino embryonic antigen (CEA) levels [168]. Local treatment of cancer patients with BIS-1 IgG BsAbs was associated with a local inflammatory reaction in which polymorphonuclear cell infiltrates and ele-

vated cytokine levels, such as TNF-α and IL-6, could be seen at the site of the treatment. In addition, elevated levels of soluble CD8 were detectable early after the start of the treatment, suggestive of an active role of T lymphocytes. Similar results have been described upon i.p. injection of ex vivo activated T-cells that were preincubated with a CD3× FBP-reactive BsAb, OCTR [61,188]. In this multicentre study, a 27% i.p. response rate was achieved [169]. Specific lysis of FBP-positive tumor cells was accompanied by accumulation of lymphocytes and macrophages at the site of treatment. It is possible that BsAb unrestricted anti-tumor activity could also be induced because of the presence of up-regulated adhesion molecules on tumor cells [168].

Besides cytokine profiles and toxicity, other signs of effective immune function have been observed. In vivo binding of the BsAb has been determined and quantified as the percentage of CD3 receptors occupied by the BsAbs in serial blood samples [90,167]. The percentage of CD3 receptor binding by BsAb was dose-dependent, reaching levels of 6% at doses that cause serious toxicity [167]. Despite the low percentage of occupied CD3 receptors, unbound functional BsAb remained in the circulation [172]. The percentage of CD3 occupation rapidly declined after the end of the infusion, which could be explained either by modulation of the CD3 complex or by redistribution of BsAb-loaded T-cells leaving the circulation while fresh T-cells entered the circulation. Support for the latter phenomenon was found in the observation that the in vivo decline of BsAb binding to T-cells occurred much faster than in vitro CD3 modulation of the same T-cells [172]. Free BsAb was determined in a number of studies. Circulating free BsAbs could be assessed with a serum half-life ($T_{1/2}$) ranging from ±2–12 h using an anti-FcR-based BsAb [90] to ±10 h using an anti-CD3-based BsAb [152]. No correlation between dosing and $T_{1/2}$ seemed apparent in these studies. Unlike local treatment protocols, where evaluation of anti-tumor activity is relatively simple, evidence of in vivo anti-tumor activity upon systemic application of BsAb is difficult to obtain. Despite this difficulty and the phase I character of the studies in which a number of BsAbs have been tested to date, some encouraging preliminary data suggest that effective anti-tumor activity occurred, leading to clinical improvement in two out of ten patients who were treated with a single dose of MDX-210 [90]. It should be stressed that experimental data in animal models suggest that BsAbs will be most effective in clinical situations of minimal disease [49,189]. In this respect, it is noteworthy to mention that the observed tumor regressions occurred in tumor masses smaller than 4 cm in diameter [90,168,169,188].

Ex vivo cytolytic activity has been reported using immune effector cells from the blood of patients treated with BsAbs [167,171,172]. In a whole blood ADCC assay, using blood cells from G-CSF-treated patients, it was shown that cytolytic activity was induced 30 days after the application of MDX210 [171]. This phenomenon correlated with the observation of Valone et al. [90], who described the appearance of natural anti-HER-2/*neu*-reactive antibodies weeks after the application of the same BsAb. The appearance of such antibodies might be an indication of BsAb-stimulated myeloid effector function, but could also represent ab3 antibodies arising in an anti-idiotypic network [27]. Similar results have been described in the clinical application of the EGP-2-reactive CO17-1A antibody [5,190]. In contrast to Repp et al. [171], we were not able to directly assess cytolytic activity in a whole blood assay, perhaps due to the fact that lymphocytes were targeted instead of neutrophils. However, using lymphocytes isolated directly at the end of the BsAb BIS-1 $F(ab')_2$ infusion, we were able to clearly detect BIS-1 $F(ab')_2$-directed lysis of EGP-2-positive target cells in vitro. Interestingly, the maximal cytolytic activity, assessed by adding excess BIS-1 $F(ab')_2$ to the assay, was found to have an optimum at a BIS-1 F(ab')2 dose of 3 μg/kg [167,172]. At higher dosages of BIS-1 $F(ab')_2$, decreased cytolytic activity was observed (Fig. 6), which coincided with a flow cytometrically determined preferential disappearance of cytotoxic T-lymphocytes from the blood during and after the BIS-1 $F(ab')_2$ infusion. Similar results in which LFA-1 bright CD8-positive/T-cells, representative of the cytolytic T-cell fraction [191,192], left the circulation have been described to occur during acute immune activation [193].

## 6. Prospects for future studies

Different BsAb-based treatment strategies have been described in recent literature. It may be antici-

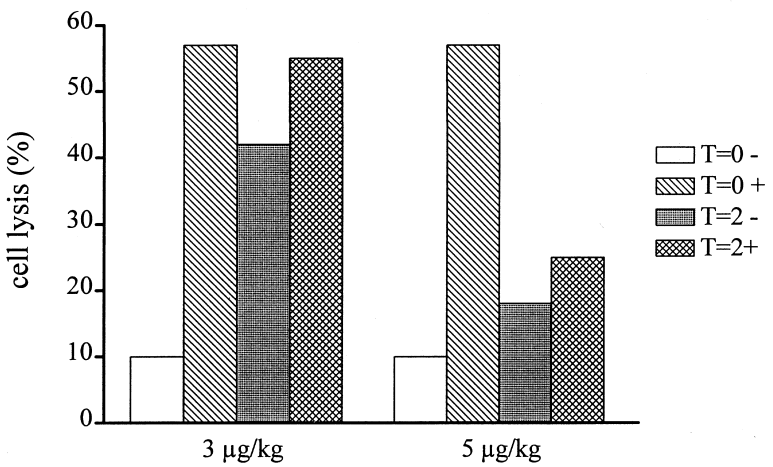


Fig. 6. In vitro killing capacity against GLC-1M13 (EGP-$2^+$) target cells of peripheral blood lymphocytes obtained from two patients just before (T = 0) and directly after (T = 2) treatment with 3 and 5 μg of BsAb BIS-1 F(ab′)$_2$/kg (CD3 × EGP-2). Cytolytic activity was assessed in the absence (T = 0 − and T = 2 − ) and presence (T = 0 + and T = 2 + ) of in vitro added 0.1 μg/ml BIS-1 F(ab′)$_2$, to assess the maximal inducible killing capacity. The data shown were obtained at an effector-to-target ratio of 100:1. Killing of EGP-$2^-$ target cells never exceeded background killing of GLC-1M13 cells (not shown). (Figure adapted from ref. [172] with permission).

pated that combinations of the different modalities discussed here might act synergistically in the eradication of established or residual tumors. However, it should be noted that the tumor itself also plays an important role in the eventual effectiveness of immunotherapeutic approaches. Heterogeneity of tumors for the expression of tumor antigens, production of immune suppressive cytokines and tumor-derived factors, such as bFGF or gangliosides, might seriously hamper successful targeted immunotherapy [194–197]. In particular, lymphomas and leukemias have been documented to suppress immune function [197,198], but also, tumor-infiltrated T-cells isolated from other tumor types often show a poor functional performance [199,200], which can, however, be reversed using the proper activation stimuli [201]. Furthermore, lymphomas and carcinomas grow in immunologically fundamentally different tissues, which imposes different needs and qualifications for the effector cells to encounter their targets. Functional FasL expression by the tumor might impose a new recently described problem for establishing an effective T-cell-based immunotherapeutic protocol [202]. Since pre-activated T-cells are relatively insensitive to the death signal delivered by FasL, extra attention should be focused on protocols to fine-tune T-cell activation and killing. It has become clear that careful dosing and proper effector cell stimulation hold the keys to successful in vivo anti-tumor activity [49]. The phenomenon of desensitization or receptor molecule down-modulation on the effector cells, and the role of costimulation as part of that, should be carefully investigated and could lead to more successful anti-cancer treatment strategies. Also, the vaccine-like effects that have been reported and discussed above form an attractive phenomenon to further investigate and exploit clinically [149,203].

From the foregoing, it may be expected that minimal residual disease represents the most favourable stage of cancer to be amenable to BsAb-mediated immunotherapy. Small cell lung carcinoma (SCLC), but also leukemias and lymphomas, hypothetically represent good candidates for adjuvant BsAb-based cellular immunotherapy. Chemotherapy and radiotherapy can induce more than 50% complete remissions in SCLC [204]. However, a relapse, in which the tumor does not respond to any conventional form of therapy, almost inevitably occurs. These patients present themselves with a relatively small tumor load and a relatively effective immune system, which might embody the most ideal setting for BsAb-mediated immunotherapeutic treatment.

One of the major problems with the introduction of immunotherapy as an effective therapeutic modality is the complexity of the system exploited. As discussed in this review, intervention in this

delicately balanced system may result in serious and unpredictable systemic side effects which, however, also reflects the power of the immune system. The challenge for future clinical application of BsAb-mediated immunotherapy is to redirect this power to its maximum against the malignant cells.

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