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# Application of Recombinant Antibodies in Cancer Patients

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## 1. Introduction

As a consequence of the invention of the hybridoma technology by Köhler and Milstein ***(1)***, many monoclonal antibodies (MAbs) have been evaluated in clinical trials since the early 1980s. Clinical outcomes were generally poor ***(2–5)***, with the notable exception of marked tumor responses, including long-term remissions of patients with malignant B-cell lymphoma who were treated with patient-specific antiidiotypic antibodies ***(6–8)***. The main factors responsible for these initial shortcomings were related to the immunogenicity of the murine protein, to modulation of targeted antigens, and to the poor ability of these antibodies to sufficiently mediate antibody-dependent effector functions in humans.

The advent of recombinant antibody technology led to an enormous revival in the use of antibodies as therapeutic agents in cancer therapy. This review provides a brief historical sketch of the development of recombinant antibodies for immunotherapy of cancer, which is followed by the most significant clinical data, as exemplified by the two clinically most established recombinant antibodies to date. Finally, we will focus on future prospects for antibody-based therapeutic concepts in oncology.

## 2. The Development of Recombinant Antibodies for Cancer Therapy

### *2.1. Chimeric Antibodies*

The first reports of the successful cloning of immunoglobulin gene segments were published in 1977 ***(9,10)***, nearly one decade after the discovery of the existence of restriction endonucleases, which enable microorganisms to cleave foreign DNA in a highly specific manner ***(11)***. It took another several years until the first recombinant antibodies were constructed as "chimeric" molecules by fusing the rearranged murine variable V(D)J gene segments of a mouse MAb to human constant domains ***(12,13)*** or were generated as a recombinant Fab fusion protein by replacing the Fc fragment with

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an enzyme moiety *(14)*. Chimerized antibodies retained the specificity of the monoclonal ancestor and proved to be immunogenic in only a very small subset of patients when administered in clinical trials *(15–19)*. Half lives of chimeric antibodies in human serum were shown to be significantly longer compared to the respective parental murine MAbs *(15,16,18,20,21)* and even increased after repetitive administrations *(18,20,21)*. Moreover, chimeric antibodies were capable of mediating antibody-dependent cellular cytotoxicity (ADCC) with human effector cells and/or to activate the complement cascade very efficiently, both in vitro *(22–25)* and in vivo *(26,27)*.

### 2.2. Humanized Antibodies

In order to further decrease the immunogenicity of murine antibodies, the first monoclonal antibody was "humanized" in 1986 by grafting the gene segments coding for the antigen binding loops onto human framework regions. Although the expressed antibody retained its full specificity, a substantial decrease of affinity was observed *(28)*. Subsequent cristallographic X-ray diffractions of many antibody variable region binding domains and computer modeling studies based on these crystal structures, allowed, with exception of loop H3, the identification of a small number of "key residues" located either in the loops itself or in the framework regions. These residues determine the main chain conformation ("canonical structure") of the antigen binding loops *(29,30)*. Based on these fundamental insights into antibody structure, antibodies were successfully modified by retaining murine residues within the acceptor framework regions *(31–33)* or by secondary directed mutagenesis to restore observed decreases in affinity after humanization *(34,35)*. More recently, antibodies were humanized by "resurfacing" the variable domains. In this case, only accessible residues are of human origin whereas buried, structure-maintaining backbone residues remain murine *(36)*.

Many chimeric and humanized antibodies have been employed in clinical trials (reviewed in **ref.** *37*) and, as a result of these studies, two cancer-specific reagents have been approved by the American Food and Drug Administration (FDA) for the treatment of non-Hodgkin's lymphoma and metastatic breast cancer, respectively.

In order to extend effector functions, chimeric or humanized antibodies were conjugated to radionuclides and drugs and successfully employed in Phase I/II clinical trials *(38–43)*. As a consequence, the first humanized antibody-drug conjugate (Gemtuzumab Ozogamicin = Myelotarg™) was recently FDA-approved as an orphan drug for the treatment of acute myeloid leukemia of patients 60 yr or older and who are not considered candidates for cytotoxic chemotherapy *(44)*.

To retarget effector cells of the immune system, bispecific chimeric or humanized antibody molecules have been developed to activate cytotoxic T cells *(45–48)* or myeloid effector cells *(49)*. The latter construct has been administered in clinical Phase I trials to patients with a variety of solid tumors *(50–52)*.

### 2.3. Recombinant Antibody Fragments

The successful expression of functional antigen-binding domains in *E. coli* *(53,54)* provided the basis for the rapid development of a new generation of antibody based

molecules with potentially great therapeutic impact. Noncovalently linked $V_H$ and $V_L$ domains tend to dissociate from each other, particularly at low protein concentrations ***(55)***. In order to stabilize the assoziation of the two domains, a synthetic linker peptide has been introduced connecting both variable domains ***(56)***. These "single-chain" molecules were shown to retain their full binding specificity and affinity ***(57)***. To further enhance the stability of these fragments, intermolecular disulfide bonds were generated by introducing cystein residues in the $V_H$ and $V_L$ framework regions, respectively, and were shown to increase the stability markedly while retaining the full antigen binding properties ***(55,58,59)***. ScFv fragments were engineered as fusion molecules to employ artificial effector functions, since they are unable to mediate natural effector functions owing to the lack of the Fc portion of whole antibodies. This was accomplished by linking them to toxins ***(60–64)*** cytotoxic ribonucleases ***(65–67)***, enzymes for activation of prodrugs ***(68–72)***, radionuclides (reviewed in **ref. *73***), cytokines ***(74,75)***, or chemokines ***(76)***. Recombinant antibody fragments have been generated as bispecific molecules by various techniques ***(77–81)*** to retarget human cytotoxic T cells ***(77,80,82–85)*** or natural killer cells ***(86)***. Several methods were employed to increase the avidity of bispecific antibody fragments by constructing them as tetravalent bispecific molecules ***(87–89)***.

One approach for combining antibody targeting and activation of cellular effector cells is the construction of chimeric receptor molecules ("T-Body"), consisting of a tumor specific single chain antibody and a signal domain for activation of a cytotoxic effector cell. Engrafting of the constructs into cytotoxic T cells results in the MHC independent destruction of scFv-targeted tumor cells ***(90–94)***.

From these third generation antibodies, recombinant immunotoxins are now beginning to enter clinical trials and initial data confirm them as very potent ***(95,96)***.

### 2.4. Phage Display-Derived Antibodies

Parallel to the rapid development of engineering the described variants of functional hybridoma derived MAbs, new methods were developed to eventually bypass hybridoma technology. In 1985 was shown that peptides could be expressed on the surface of filamentous bacteriophage. The gene fragments encoding the foreign DNA were inserted into the filamentous phage gene III in order to encode a fusion protein displayed on the surface of the phage without disrupting its capability of infection upon binding of pIII to the F pilus of the bacteria. These phages could be enriched more than 1000-fold after a single round of selection through binding of the displayed peptide to a MAb ***(97)***. In 1990 McCafferty and colleagues successfully expressed the variable domains of an antibody on the surface of filamentous phage. The phage-derived antibody retained its full binding and specificity to its antigen ***(98)***. Antibody variable gene segments of different subgroups could be amplified by the polymerase chain reaction (PCR) ***(99)***, using either degenerated primers ***(100,101)***, a set of family-specific oligonucleotides ***(102)***, or primers based on the amino acid sequences of immunoglobulin variable domains ***(103)***. This allowed the construction of antibody libraries by cloning the PCR-amplified $V_H$ and $V_L$ repertoire of B lymphocytes into suitable phagemid vectors ***(104–106)*** for expression and screening of randomly asso-

ciated variable domain fragments on the phage surface. Binding phage antibodies were isolated from a large number of nonbinders by enrichment of the particles through multiple rounds of in vitro-panning against the antigen of choice and extensive washing steps to remove nonbinders. Phage antibody technology has since become the most powerful tool for isolating highly specific antibodies with high affinities to predefined antigens. Antibody libraries have been constructed from patients ***(107–109)***, immunized mice ***(110–112)***, as naive libraries from (multiple) healthy donors ***(113–116)***, and as (semi)synthetic libraries by randomizing sequences in one or more hypervariable regions ***(117–123)***. Antibody repertoires were recently also displayed on ribosomes ***(124,125)***, allowing for the generation of very large libraries to be screened within a short period of time.

The natural antibody repertoire of camels and other camelid species contains a large number of functional antibodies devoid of a light chain ***(126)***. A phage display library from the $V_H$ repertoire of an immunized camel has been constructed ***(127)*** and single $V_H$ domain antibodies with subnanomolar affinities were isolated ***(128)***.

Functional single $V_H$ domains with high affinities have recently also been isolated from a human $V_H$ repertoire phage display library ***(129)***.

Phage display-derived antibody fragments have begun to be introduced in clinical trials as radioimaging reagents in cancer patients ***(130,131)***.

### 2.5. Recombinant Antibodies from Transgenic Mice

Many attempts to generate human antibodies by employing the hybridoma technology have been unsuccessful, mainly from the lack of a suitable human myeloma cell line to immortilize B cells (reviewed in **ref.** ***132***). Alternately, human antibodies were produced in transgenic mice by replacing the murine immunoglobulin loci of the host genome with the respective human counterpart ***(133–137)***. Hyperimmunization of the transgenic animals with (tumor) antigens of choice results in the clonal activation of B lymphocytes producing human antibodies. Upon rechallenge of the mice with the antigen of interest, affinity maturated antibodies can be generated in vivo. Immortalization of B cells expressing these antibodies can be achieved by standard hybridoma technology, resulting in the production of entirely human antibodies from established cell lines.

## 3. Clinical Data

It took more than 10 years from the initial development of the first generation of recombinant antibodies to become an integrated part of treatment concepts in oncology today.

### 3.1. Rituximab (Rituxan™, Mabthera™)

The chimeric antibody Rituximab (Rituxan™, Mabthera™) binds to the transmembrane antigen CD20, which is strongly overexpressed in most B cell lymphomas ***(138)***. In two independent Phase II clinical multicenter studies, Rituximab has been administered to more than 200 patients with refractory or relapsed low grade B-cell non-Hodgkin's lymphoma (B-NHL) in four weekly doses of 375 mg/m$^2$. Overall response

rates in 185 evaluable patients were around 50% with complete remissions of 9% and 6%, respectively, by a medium time to progression of 10.2 and 13 mo, respectively ***(18,139)***. A favorable tumor response was associated with a histology of follicular NHL, sustained high serum levels of antibody after the first infusion, and a longer remission after prior chemotherapy ***(18)***. Treatment-related side effects, mostly observed in the first course of treatment, were low and reversible, and in most cases consisted of fever, chills and headache. These side effects were usually reversible by merely lowering the infusion rate. Only two patients developed an antibody response against the chimeric antibody. Most patients exhibited increasing serum concentrations of the chimeric antibody throughout the treatment courses, associated with progressively longer half lives from 76.3 h to 205.8 h after the fourth infusion ***(21)***. The impressive results of these clinical trials led to the FDA approval of Rituximab in 1997 as the first recombinant antibody for tumor therapy.

Rituximab proved its potency also on patients with intermediate and high grade B-NHL. In a Phase II study of 54 patients with relapsing intermediate- and high-grade lymphomas single-agent therapy with Rituximab achieved 5 complete and 12 partial responses. Patients with diffuse large B-cell lymphoma achieved a favorable response rate of 37%, and the median time to progression exceeded 246 d for the responding patients ***(140)***. These results formed the basis of a randomized trial in elderly patients (60–80 yr of age) with diffuse large B-cell lymphoma, which compared 8 cycles of a three-weekly CHOP regimen with the same chemotherapy plus Rituximab 375 mg/m$^2$ given on day one of each CHOP cycle. The combination of CHOP and Rituximab reduced the rate of primary progressions by 17%. After a median time of observation of only 12 mo in 328 evaluable patients, event-free and overall-survival achieved with CHOP + Rituximab was significantly better than that after chemotherapy only ***(141)***. Patients of the low and low-intermediate risk group according to the international prognostic index (IPI) profited more from Rituximab than patients in the high and high-intermediate risk group ***(142)***. Ongoing trials will have to show whether these results can be confirmed in younger patients of the different risk groups. In a recently conducted Phase II clinical trial, similar encouraging results for the combination of Rituximab with standard CHOP chemotherapy in 33 previously untreated patients with high grade B-NHL were achieved. In this study 6 cycles CHOP at 3-wk intervals plus Rituximab 2 d prior to each chemotherapy course were administered. The overall response rate was 94% including 61% complete remissions and the median duration of response and time to progression had not been reached after a median observation time of 26 mo. 29 of 31 responding patients remained in remission during this follow-up period. No additional toxicity has been observed compared to patients when treated with CHOP alone ***(143)***.

Good response rates with long lasting remissions were recently also reported for patients with indolent low-grade B-NHL being treated with Rituximab/CHOP in combination ***(144)***. In contrast to chemotherapy alone, combined immunochemotherapy induced molecular remissions in some patients with follicular lymphoma. In those patients, the initial detection of bcl-2 translocation transcripts by PCR in bone marrow cells changed to bcl-2 negativity after treatment ***(144)***. Favorable response rates

observed in patients with less advanced stages of the disease suggested a most promising role of Rituximab in eradicating minimal residue disease in patients with low tumor burden. To prove this hypothesis, 49 patients with follicular B-NHL and low tumor burden were treated with 4 weekly infusions of Rituximab (375 mg/m$^2$) as a single-agent first-line therapy. The response rate was 73% including 20% complete remissions in 49 evaluable patients 1 mo after treatment. Molecular remissions in the bone marrow were observed in 31% of the patients on d 50 and were positively correlated to progression-free survival ***(145)***.

Current studies suggest other important roles for Rituximab in radioimmunotherapy ***(38)*** and for the in vivo purging of tumor cells from hematopoetic stem cells prior to high dose chemotherapy and subsequent autologous stem cell transplantation ***(146)***.

### 3.2. Trastuzumab (Herceptin™)

Growth factor receptors play an important role in the regulation of epithelial cell growth. In epithelial cancers dysregulation of these receptors is a common feature in the pathogenesis. The protooncogene Her-2 encodes the 185 kDa transmembrane glycoprotein receptor p185$^{Her-2}$ with intrinsic tyrosine kinase activity and is highly homologous to the epidermal growth factor receptor family (ECFR) ***(147)***. This protein is expressed in a variety of solid tumors including breast, lung, prostate and gastric cancer. In breast cancer p185$^{Her-2}$ is expressed in more than 25% of the cases and is associated with a poor prognosis ***(148,149)***. 4D5 is a MAb binding to p185$^{Her-2}$ and interferes with growth factor receptor-mediated growth stimulation. In preclinical trials the antibody has been capable of inhibiting tumor growth in vitro ***(150)*** and in a breast cancer xenograft mouse model ***(151)***. 4D5 has been humanized in order to reduce its immunogenicity and to enhance the capability to mediate additional toxicity via natural effector functions ***(152)***. This antibody, termed Trastuzumab (Herceptin™), was employed in a Phase II clinical trial as a single agent in 46 patients with p185$^{Her-2}$ overexpressing metastatic breast cancer. Most of the patients had been heavily pretreated and the reagent was administered with a loading dose of 250 mg, followed by 10 weekly doses of 100 mg. Patients without disease progression received a weekly maintenance dose of the antibody of 100 mg. The overall response rate was 11,6% in 43 evaluable patients including one complete remission and four partial responses. Toxicity consisted mainly of fever and chills, but no severe side effects were observed. No antibodies directed against the humanized antibody could be detected ***(153)***. In a multinational Phase II clinical trial, 222 extensively pretreated patients with advanced p185$^{Her-2}$ expressing metastatic breast cancer were enrolled. Patients received a loading dose of 4 mg/kg, followed by weekly infusions of 2 mg/kg antibody. In this study a 15% overall response rate with 8 complete and 26 partial remissions was observed in 213 evaluable patients. The median duration of response was 9.1 mo and the median duration of survival was 13 mo. Although higher response rates up to 49% have been reported for second line therapy with docetaxel in anthracycline resistant patients ***(154)***, the median duration of survival (10 mo) was actually shorter compared to patients treated with Trastuzumab (13 mo). Patients with high expression of p185$^{Her-2}$ and patients who relapsed more than 6 mo after prior chemotherapy showed higher

response rates and a longer time to disease progression. The mean half life of the antibody was 6,2 d. Significant side effects were reported by 41% of the patients, consisting of pain, asthenia, fever, chills, nausea, and vomiting as the most frequent events. These side effects were reversible and occurred almost exclusively in the first cycle of treatment. In addition, the study reported serious cardiac dysfunction in a total of almost 5% of the patients, manifesting as congestive heart failure, cardiomyopathy and/or decrease in cardiac ejection fraction by more than 10%. Nine out of ten of these patients had previously received chemotherapies containing anthracyclines, and had one or more risk factors for anthracycline induced cardiomyopathy, such as cumulative doxorubicin dose of more than 400 mg/m$^2$, previous radiotherapy to the left chest, age over 70 yr or history of hypertension. This study also included a quality-of-life assessment, surveying the variables physical function, role function, social function, global quality-of-life and fatigue. Patients who responded to therapy reported improvements in all of the evaluated parameters ***(155)***. Based on these results Trastuzumab was approved by the American FDA for treating patients with p185$^{Her-2}$ overexpressing metastatic breast cancer in relapse.

Preclinical data suggested synergistic effects of Herceptin in combination with various chemotherapeutic agents in tumor xenografted athymic mice ***(156,157)***. In a Phase III multinational clinical trial Trastuzumab has been administered to 464 previously untreated patients with metastatic p185$^{Her-2}$ positive breast cancer either as a single agent or in combination with either doxorubicin plus cyclophosphamide or paclitaxel. The combination therapy significantly increased the overall reponse rates from 32% to 50% and prolonged the median time to progression from 6.1 (doxorubicin plus cyclophosphamide alone) to 7.8 mo (doxorubicin plus cyclophosphamide plus trastuzumab) and from 3.0 (paclitaxel alone) to 6.9 mo (paclitaxel plus trastuzumab), respectively. The relative risk of death could be reduced by 20% at a median follow-up of 30 mo. No patient developed antibodies against Trastuzumab. Adverse side effects were generally mild to moderate in severity and occurred more frequently in the combination therapy groups. The most severe side effect consisted of WHO grade 3–4 cardiac toxicity, most pronounced in the Trastuzumab/doxorubicin/cyclophosphamide therapy group (27%) and less common and severe in patients treated with doxorubicin/cyclophospamide without Trastuzumab (8%) or in the combination therapy group with Trastuzumab/paclitaxel (13%) or paclitaxel alone (1%) ***(158)***. These data clearly provide evidence for synergistic toxicity of Trastuzumab in combination with cytostatic drugs by yet unknown mechanisms.

The role of Herceptin in an adjuvant setting is currently under investigation in a Phase III clinical trial ***(159)***.

## 4. Future Prospects

### *4.1. HAMA Response*

One of the major limitations in using monoclonal antibodies as therapeutic agents for treating cancer has been the immnunogenicity of murine antibodies. The development of human anti-mouse antibodies (HAMA) in patients treated with these reagents

generally precluded repeated administrations due to allergic reactions and rapid elimination of the murine protein. Chimeric, humanized, and fully human recombinant antibodies or antibody fragment derivatives thereof were shown to reduce immunogenicity dramatically. However, the issue of immunogenicity is not understood in detail. It still remains unclear why the development of a HAMA response is not occurring in all patients treated with monoclonal murine antibodies. Moreover, the HAMA response is strongly variable for different MAbs and not always associated with an unfavorable clinical outcome ***(6,8,160,161,162,163)***. According to Jerne's network hypothesis ***(164)*** the HAMA response has been proposed as being potentially beneficial for patients due to the generation of anti-idiotypic antibodies, eliciting a humoral and/or cellular immune response to the tumor in the recipients ***(160)***. In a number of clinical trials involving small groups of patients, clinically favorable outcomes were attributed to vaccination effects generated by the HAMA response ***(165–169)***. However, in a recent large randomized, multicenter clinical Phase III study involving colorectal carcinoma patients treated with the monoclonal 17-1A in an adjuvant setting, neither a positive or negative correlation between the development of a HAMA response and the clinical outcome could be observed ***(163)***. Moreover, in large clinical trials with chimeric and humanized antibodies in cancer patients the development of antiidiotypic antibodies in treated patients was only rarely observed ***(18,140,143,144,155,158)***, although the generation of anti-idiotypic antibodies should be focused to the murine residues of the antigen-binding domains.

### 4.2. Selection of Target Antigens

The ultimate goal in cancer therapy is the complete destruction of the tumor while sparing healthy tissue. These issues, efficiency and selectivity, have been extensively addressed in antibody-based therapeutic approaches ever since the first patient was treated with a MAb in 1979 ***(170)***. Various factors have since been identified as influential to the success of immunotherapy with monoclonal and recombinant antibodies.

The selection of an appropriate target antigen is one of the most essential prerequisites in the employment of antibodies as therapeutic agents. The target antigen should be expressed on the malignant cells selectively, consistently, and with high density. A variety of cell surface antigens have been used as targets for therapeutic antibodies. Early clinical studies reported antigen modulations after antibody administration, thereby vacating therapy ***(2,171,172)***. With the exception of patient-specific anti-idiotypic antibodies directed against clonally expressed immunoglobulin on lymphoma cells, target antigens are generally not tumor-specific but also expressed on subsets of normal cells. The overexpression of cell-surface antigens, physiologically involved in cell-growth regulation, represents a complex role of these molecules in the tumor pathogenesis and the underlying molecular mechanisms are often only poorly understood. Thus the effects antibodies trigger upon binding to these receptors is hardly predictable. Only recently has it become possible to identify truly tumor specific antigens through either the SEREX technology ***(173)*** or by methods of antibody phage display technology ***(174–176)***. These antigens represent a novel class of most promising targets for antibody-based therapeutics.

### 4.3. "Classical" Effector Functions Mediated by Recombinant Antibodies

Murine MAbs of IgG2a isotype are capable to activate human complement and/or mediate antibody-dependent cytotoxicity (ADCC) ***(177–182)***. This capability, however, is often quite limited and additionally hampered by the HAMA response leading to rapid neutralization and degradation of the murine protein. In contrast, recombinant antibodies mediate these "classical" effector functions much more effectively than the murine counterpart ***(22,183,184)***. Based on the impressive clinical results observed in patients who were treated with Rituximab and Trastuzumab, the underlying mechanisms of "classical" and antibody-specific effector functions have been examined more in detail.

For Rituximab it has been shown that high coexpression of the cell surface complement inhibitors CD55 and CD59 on the malignant cell could abolish complement dependent cytotoxicity in vitro almost completely. Blocking of these molecules with MAbs resulted in restoration of complement-mediated cytotoxicity ***(25)***.

The efficiacy of ADCC in vivo is largely dependent on the interaction of activating FcγRIII and inhibiting FcγRIIb receptors expressed on myeloid cells (reviewed in **refs.** ***185,186***). Mice deficient in the common γ chain ($FcR\gamma^{-/-}$), thus lacking the activating FcγRI/FcγRIII, and mice without the inhibiting FcγRIIb, were each mated with athymic nude mice for use in $CD20^{+}$ or $p185^{Her\text{-}2}$ expressing human xenograft tumor models. In $FcR\gamma^{+/+}$ mice, the tumor mass of established $p185^{Her\text{-}2}$ expressing carcinomas could be reduced by 96% and 90%, respectively, when treated with Trastuzumab or its murine ancestor 4D5. Similarly, tumor size of xenotransplanted CD20+ lymphomas in $FcR\gamma^{+/+}$ mice could be reduced by >99% by treatment with Rituximab. These effects could be enhanced in both tumor models in $FcR\gamma IIB^{-/-}$ deficient xenotransplanted mice. In contrast, $FcR\gamma^{-/-}$ mice developed palpable tumors in both tumor models in almost all cases. To further investigate the role of Fc receptors, 4D5 was systematically mutated to disrupt Fc binding of the antibodies to its receptors. The resulting mutant retained the wild type characteristics of its half live in vivo, antigen binding properties, and $p185^{Her\text{-}2}$ receptor blockade. However, all mice treated with the mutant antibody developed palpable tumors ***(27)***. These results clearly demonstrate the crucial role of Fcγ receptor interactions with Fc fragments for the in vivo efficacy of recombinant antibodies.

### 4.4. Antibody-Specific Effector Functions

Besides the importance of successful recruitment of "classical"effector functions, some antibodies were shown to be capable of mediating tumor cell killing by interfering with cell-signaling pathways. Binding of natural ligands to cell-surface receptors can mediate signal transduction events by activation of protein kinases and phosphatases leading to the release of a second messenger and the subsequent transcription of genes involved in cell growth regulation and apoptosis. Ligation of cell-surface receptors with MAbs could mimic the natural ligand of these cell-surface receptors, thereby triggering signal-transduction events (reviewed in **ref.** ***187***). It has recently been reported that CD20 ligation with Rituximab activates the protein tyrosine kinases

(PTK) Lyn and Lck leading to downstream activation of PTK substrates, such as phospholipase C. Activation of these substrates increases intracellular calcium levels, which in turn might activate the caspase cascade directly or provide further downstream signals leading to subsequent apoptosis ***(188,189)***. Enhancement of apoptosis could be achieved by crosslinking Rituximab with either secondary antibodies or Fc receptor-bearing cells ***(188)***. Furthermore, hypercrosslinking of antigens with MAb homodimers could mediate tumor cell $G_o/G_1$ arrest or apoptosis very efficiently by not yet well-defined mechanisms ***(190)***.

CD40, a member of the tumor necrosis factor receptor family, is essential for activating antigen presenting cells (APC) ***(191,192)***. Ligation of CD40 with MAbs was recently shown to be capable of "priming" cytotoxic $CD8^+$ T cells independent of T-helper cells, leading to complete eradication of $CD40^+$ lymphomas in a syngeneic mouse model. Moreover, treated mice were protected upon rechallenge with tumor cells, suggesting a role of anti-CD40 antibodies as a vaccine. The authors suggest a mechanism by which crosslinking of CD40 with the antibody may stimulate neoplastic B cells to become effective APC and present processed tumor antigens to autologous cytotoxic T cells ***(193)***.

Recent studies in some cases elucidated the molecular basis of clinically observed synergistic effects of MAbs conjugated to drugs or radionuclides. Treatment of $p185^{Her\text{-}2}$ expressing tumor cells with cisplatin followed by Trastuzumab blocked the removal of cisplatin-induced adducts by upregulation of p21/WAF1, an important mediator of DNA repair. This effect was most pronounced when cells were incubated with Herceptin in close temporal proximity to the treatment with cisplatin ***(157)***. More recently, Trastuzumab was shown also to enhance radiosensitivity of $p185^{Her\text{-}2}$ expressing cells in a time-dependent manner, possibly due to p21WAF1 dysregulation ***(194)***. These results suggest an interaction between signaling events triggered by the antibody and DNA repair pathways, thus underlining the importance of elucidating molecular mechanisms of drug interactions as crucial for optimizing administration schedules in chemo- and radioimmunotherapy regimens.

### 4.5. Biodistribution and Pharmacokinetics

The efficiacy of tumor targeting by antibody-based molecules in vivo is not only dependent on the targeted tumor antigen but also on the tumor characteristics, e.g., tumor type (hematological malignancies or solid tumors); tumor mass; accessibility and density of the target antigen; and on characteristics of the antibody molecule itself, e.g., size, charge, affinity, and avidity ***(195–197)***.

Pharmacokinetics describes the temporal sequence of the distribution and metabolism of a molecule in the body. Molecules with low molecular weights undergo ultrafiltration from the plasma in the glomerulum of the kidney. The passage of molecules across the glomerular filtration barrier decreases progressively with increasing molecular size. In addition to the permeability of the glomerular filter the molecular charge of a molecule affects its clearance. Negatively charged molecules are retarded by repulsion from the negatively charged endothelium and glomerular basement membranes of the kidney ***(198,199)***.

Intact chimeric and humanized IgG molecules (150 kDa) persist in the circulation for several days ***(21,155)*** and half lives are even prolonged after repetetive administrations ***(18,20,21)***. The rapid clearance of murine antibodies can largely be attributed to the HAMA response which leads to rapid degradation of the immune complexes. Sequences in the CH2 and CH3 regions of IgG have been shown to regulate the rate of clearance through their interaction with the neonatal Fc receptor (FcRn) ***(200)***, thus playing a most important role in "recycling" antibodies from the bloodstream ***(201–203)***. However, catabolism of antibodies is not exclusively regulated by FcRn as shown for chimeric antibodies by constant domain shuffling ***(204)***.

Antibody fragments lacking a constant domain such as scFv (27 kDa), (scFv')2 (55 kDa), Fab' (55 kDa) and diabodies (50 kDa) are cleared from the circulation more rapidly ***(89,205–207)***. However, compared to whole IgGs, small antibody fragments have a more favorable tumor penetration capacity. The comparison of tumor penetration properties of a radiolabeled single-chain Fv with larger immunglobulin forms (IgG, $F(ab')_2$ and Fab'), derived from a MAb directed against the human pancarcinoma antigen TAG-72, showed a rapid and uniform tumor penetration for the scFv in human colon carcinoma xenografts in mice ***(208)***. In contrast, a relatively restricted penetration pattern was observed for the $F(ab')_2$ (100 kDa) and the Fab' (55 kDa) fragments, respectively. Intact IgG molecules barely exceeded the perivascular regions of the tumor even at 24 h after administration ***(208–210)*** and intratumoral diffusion distances of IgGs in solid tumor tissue of only about 1 mm in 2 d were reported ***(211)***. In contrast, small antibody molecules were retained at relatively low levels but with much higher specificity at the tumor site ***(205,207,212,213)***. To address the short half lives of antibody fragments, they were chemically modified by conjugation to monomethoxypolyethylene glycol (PEG) ***(214–216)***. In the latter approach two PEG molecules were site-specifically attached to two hinge cysteine residues of an engineered Fab fragment. This resulted in a dramatic increase in the overall half life in rats and monkeys. Given the fact that PEG is of low immunogenicity ***(217)***, this substance provides a valuable reagent in order to overcome the disadvantage of short half lives of antibody fragments when administered to humans.

Affinity describes the interaction of an antibody with its antigen, and plays an important role in the humoral immune response and affinity maturation. High-affinity antibodies can be generated by phage-display technology, and natural affinity maturation can be mimicked by various techniques of recombinant antibody technology such as chain shuffling ***(218)*** or site-directed mutagenesis ***(219,220)***. The affinity of a phage display-derived scFv C6.5 with moderate affinity ($1.6 \times 10^{-8}$ *M*) against the $p185^{Her\text{-}2}$ antigen ***(221)*** could be enhanced sixfold ($2.5 \times 10^{-9}$ *M*) by light-chain shuffling, an affinity comparable to that of the hybridoma derived antibody against the same antigen ***(222)***. By sequential site-directed mutagenisis of the CDR3 region of the heavy (VH) and light chain (VL) of the same scFv, different affinity mutants were generated with up to 1230-fold higher affinities ($1.3 \times 10^{-11}$ *M*) compared to the wild-type scFv C6.5 ***(220)***. The in vivo performance of the anti-$p185^{Her\text{-}2}$-scFv mutants in tumor bearing mice supported the concept that higher affinity scFvs could enrich in solid tumors much better than mutants with lower affinity ***(197,223)***. Contrary to the

theory that high affinity antibodies are preferable for successful tumor targeting in vivo there is evidence of an existing physical penetration barrier for antibody-based molecules with extremely high affinities. The term "binding site barrier" effect was first postulated by Weinstein and colleagues ***(224)*** describing the theory that a strong binding of high-affinity antibodies confined mainly to the periphery of the tumor, might thereby prevent a deeper tumor penetration. In the meantime this theory could be experimentally validated for antibody fragments with extremely high affinities ($10^{-11}$ *M*) ***(225)***, thus questioning their values as favorable therapeutics when rapid and uniform tumor penetration is required.

Naturally occurring antibodies differ from recombinant antibody fragments in their valency of antigen binding. The multi-valency of natural antibodies contributes to an increased functional affinity from simultaneous binding to the targeted antigen epitopes, the avidity effect ***(226)***. As a consequence, multivalency has a significant influence on the dissociation kinetics, which is of particular importance under nonequilibrium conditions of antibody–antigen interactions. Multivalent recombinant antibody fragments with increased avidity were shown to enhance tumor targeting much more efficient compared to monovalent counterparts ***(73,207,213,227–229)***, thus providing a promising role for these constructs as novel therapeutic agents with improved biodistribution characteristics and pharmacokinetics.

## 5. Concluding Remarks

In conclusion, recombinant antibody technology has provided the basis for the success of immunotherapeutic reagents in oncology today. These constructs have overcome some of the major limitations previously associated with murine MAbs when administered to cancer patients. In particular, first and second generation of these reagents now employed in larger clinical trials were shown to be immunogenic in only very rare cases. In addition, "classical" effector functions were shown to be mediated much more efficiently than by murine equivalents. A number of antibodies are able to kill tumor cells not only by "classical" effector functions but also by specific interference with cell signaling. This effect can often be enhanced by multimerization of the antigen-binding domains. It remains a task of critical importance to elucidate the molecular mechanisms of how tumor associated antigens are involved in cell-growth regulation, and the effects antibodies mediate after binding to these antigens in order to utilize this knowledge for the deductive design of therapeutic antibodies.

Clinical studies employing recombinant antibodies in cancer patients generally reported very low toxicities. However, these reagents could ocassionally mediate unexpected toxicities for yet unknown reasons. These side effects need to be monitored very carefully and possible molecular mechanisms need to be investigated.

Great progress has been made in identifying novel, truly tumor specific antigens. Isolating human antibody fragments from antibody phage display libraries by panning on novel tumor specific antigens will play a key role in the development of a new generation of highly promising reagents for cancer therapy.

It is only now that the third generation of antibodies and antibody fragments enters clinical trials. Based on preclinical data, antibody fragments can be expected to have

an improved capability of penetrating solid tumors. On the other hand they do have very short half lives, which appears to be associated with a rapid first-pass clearance due to their smaller sizes. Engineering of these molecules to determine the optimized ratio between tumor retention and metabolism promises to provide the basis for improved antibody based reagents within the next few years.

In our opinion, the most promising role for antibody based therapeutics in oncology in the future will be the employment of combinations of (multivalent) molecules that target different epitopes, thus mediating different immunological effector functions and/or interfering with different growth mechanisms of the malignant cells.

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