Cancer therapeutic antibodies come of age: Targeting minimal residual disease

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ABSTRACT

Ten years after the first clinical application of Rituximab, an anti-CD20 recombinant monoclonal antibody, immunotherapy has become common practice in oncology wards. Thanks to the great diversity of the immune system and the powerful methodology of genetic engineering, the pharmacologic potential of antibody-based therapy is far from exhaustion. The recent application of Trastuzumab, an antibody to a receptor tyrosine kinase, in adjuvant breast cancer therapy marks the beginning of a new phase in cancer treatment. Here we discuss molecular mechanisms of antibody-based therapy, the emerging ability to target minimal disease and the therapeutic potential of combining antibodies with other modalities.

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1. Introduction

The vision of antibody-based therapies tracks back to the “magic bullet” concept proposed by the German chemist Paul Ehrlich, exactly 100 years ago. Accordingly, selective targeting of a toxin to a disease-causing agent will enable eradication of the disease. This vision materialized with the generation of the first monoclonal antibodies by Georges Köhler and César Milstein in 1975 (Köhler and Milstein, 1975) (see Timeline). By selecting a clone of lymphocytes that permanently secrete antibodies to a single determinant on an antigen of choice, mAbs provide the ultimate selective vector Paul Ehrlich had envisioned. Surprisingly, and in contrast to the original theory, in many cases a toxic payload need not arm mAbs in order to kill pathogenic targets. For example, the laboratory of Robert A. Weinberg identified in the early 1980s a cell surface glycoprotein, p185-Neu/HER2, which undergoes mutagenesis when animals are exposed to a chemical carcinogen (Bargmann et al., 1986). The mutant protein, which turned out to be a receptor tyrosine kinase, potently transforms naïve cells to become tumorigenic, but murine mAbs specific to p185-Neu/HER2 retarded tumorigenic cell growth in animals (Drebin et al., 1986).

The murine origin of mAbs to p185-Neu/HER2 and other antigens limits their clinical application in several respects: such immunoglobulin molecules are immunogenic when injected into humans, which shortens their half-lives in the circulation. In addition, due to their rodent constant regions, the ability of murine mAbs to recruit effector functions of the human immune system is inefficient. The first strategy that partly overcomes these limitations was introduced in 1984 (Boulianne et al., 1984; Morrison et al., 1984): the antigen-binding variable...
domains of the heavy and light chains of a parental murine antibody were, respectively, joined to human constant domains, corresponding to the heavy and light chains of immunoglobulins (see Box 1). An alternative to the generation of such mouse–human chimeric antibodies was put forward in 1988 by Greg Winter and colleagues (Riechmann et al., 1988), who humanized antibodies by grafting the murine antigen-binding loops (complementary-determining regions; CDRs) into a human immunoglobulin molecule. Phage display technologies (Marks et al., 1991; Griffiths et al., 1993) offer yet another alternative, whereby the antibody repertoire of human B cells is represented by very large gene libraries. Once selected, the affinity of the human antibody may be enhanced for clinical applications by using mutagenesis. Last, transgenic mice whose immunoglobulin loci have been genetically inactivated, and which instead carry many, or all, human immunoglobulin genes, may be used for immunization and standard hybridoma technology (Fishwild et al., 1996; Mendez et al., 1997). The first fully human antibody derived from transgenic mice, Panitumumab, an antibody to a sibling of p185-Neu-HER2, namely the epidermal growth factor receptor (EGFR) (Yang et al., 1999) has recently been approved for the treatment of colorectal cancer. Although cancer therapeutic antibodies have yet to achieve the ultimate goal of curing cancer, the number of antibodies approved for cancer therapy is steadily increasing (see Table 1), along with the mushrooming of antibodies currently tested in clinical trials (Table 2).

2. The targets for antibody-based therapy of cancer: tumor-associated antigens

Of all parameters needed for optimization of a therapeutic antibody, selection of the target antigen is the most critical step. Glycoproteins found on the surface of tumor cells emerge as useful targets for antibody-based therapy. Several tumor-associated antigens (TAAs) of hematopoietic cells have been targeted in animal or in clinical trials, including CD20, CD22, CD25, CD33, CD40 and CD52. Likewise, receptor tyrosine kinases residing on the surface of malignant epithelial cells (e.g., HER2/ErbB-2 and EGFR), or normal endothelial cells (vascular endothelial growth factor receptor; VEGFR), prove useful as targets for therapeutic antibodies (see Tables 1 and 2). A third group of antigens, which may mount an effective therapeutic response, comprises cell adhesion molecules like mucin 1 (MUC1), carcinoembryonic antigen (CEA1), various integrins (e.g., αVβ3, a molecule enriched on vascular endothelial cells) and EpCAM. The latter is the target of Panorex (Edrecolomab), a murine antibody which was used in Europe for the treatment of Duke’s colorectal cancer. EpCAM is an epithelial cell adhesion molecule, acting by mechanically attaching cells to the matrix or to other cells (Balzar et al., 1999).

This review will concentrate primarily on two antigens, CD20 and ErbB-2/HER2, as well as on antibodies that therapeutically target them. CD20 is a surface phosphoprotein, whose biology is poorly understood, despite the remarkable success of immunotherapy targeted at this antigen (Cragg et al., 2005). For example, no ligand of CD20 has been identified and mice defective in the corresponding genes display an apparently normal phenotype (O’Keefe et al., 1998). The 37 kDa protein is expressed on 95% of cases of B cell lymphoma and leukemia, and no soluble form of the antigen has been detected, making it an attractive target for therapeutic mAbs. CD20 spans the plasma membrane four times and presents an extracellular loop of 43 residues, between the third and the fourth transmembrane domains (Poljak and Deans, 2002). It is thought that CD20 exists as a tetramer, which translocates upon antibody binding into lipid-ordered membrane domains called rafts. Following ligation of B-cell antigen
receptors, raft-localized CD20 may act as a store-operated
calcium channel (Polyak et al., 1998). Antibody-induced asso-
ciation of CD20 with lipid rafts and consequent calcium influx
and caspase activation, may play essential roles in immuno-
therapy (Janas et al., 2005), as will be discussed below.

Unlike CD20, ErbB-2/HER2 is a large (1234 residues, 185 kDa)
glycoprotein that spans the plasma membrane only once. The
extracellular portion of human ErbB-2 (residues 1–632), like the
EGFR, consists of four sub-domains, which present multiple
antigenic determinants, including the juxtamembrane Trastu-
zumab’s epitope (Cho et al., 2003). Unlike EGFR and other ErbB
family members, no known growth factor binds with the
extracellular domain of ErbB-2. Instead, this receptor tyrosine
kinase acts as a shared signaling subunit that heterodimerizes

Table 1 – Recombinant antibodies approved for use in oncology

<table>
<thead>
<tr>
<th>Antibody trade name (generic name)</th>
<th>Antigen target</th>
<th>Antibody type</th>
<th>Strategy to enhance activity of naked antibody</th>
<th>Tumor target</th>
<th>Approved in</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rituxan (Rituximab)</td>
<td>CD20</td>
<td>Chimeric IgG1</td>
<td>Combination with chemotherapy</td>
<td>NHL</td>
<td>1997</td>
</tr>
<tr>
<td>Herceptin (Trastuzumab)</td>
<td>ErbB-2</td>
<td>Humanized IgG1</td>
<td>Combination with chemotherapy</td>
<td>Metastatic breast cancer</td>
<td>1998</td>
</tr>
<tr>
<td>Mylotarg (Gemtuzumab ozogamicin)</td>
<td>CD33</td>
<td>Humanized IgG4</td>
<td>Calicheamicin conjugate</td>
<td>AML</td>
<td>2000</td>
</tr>
<tr>
<td>Campath (Alemtuzumab)</td>
<td>CD52</td>
<td>Humanized IgG1</td>
<td>None</td>
<td>B-cell CLL</td>
<td>2001</td>
</tr>
<tr>
<td>Zevalin (Ebritumomab tituxetan)</td>
<td>CD20</td>
<td>Murine IgG1</td>
<td>90Yttrium</td>
<td>NHL</td>
<td>2001</td>
</tr>
<tr>
<td>Bexxar (131I-Tositumomab)</td>
<td>CD20</td>
<td>Murine IgG2</td>
<td>131Iodine</td>
<td>NHL</td>
<td>2001</td>
</tr>
<tr>
<td>Avastin (Bevacizumab)</td>
<td>VEGF</td>
<td>Humanized IgG1</td>
<td>Combination with 5-fluorouracil</td>
<td>CRC</td>
<td>2004</td>
</tr>
<tr>
<td>Erbitux (Cetuximab)</td>
<td>EGFR</td>
<td>Chimeric IgG1</td>
<td>Combination with irinotecan (CRC) or radiotherapy (SCHN)</td>
<td>CRC</td>
<td>2004/2006</td>
</tr>
</tbody>
</table>

AML, acute myelogenous leukemia; BC, breast cancer; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; IFL, irinotecan/leucovorin/5-fluorouracil; NHL, non-Hodgkin’s lymphoma; SCHN, squamous-cell of head and neck.
Table 2 – Recombinant antibodies in clinical evaluation for use in oncology (see www.clinicaltrials.gov)

<table>
<thead>
<tr>
<th>Antibody trade name (generic name)</th>
<th>Antigen target</th>
<th>Antibody type</th>
<th>Strategy to enhance activity of naked antibody</th>
<th>Tumor target</th>
<th>Clinical trial</th>
</tr>
</thead>
<tbody>
<tr>
<td>BEC2 (Mitumomab)</td>
<td>Anti-idiotypic mAbs, GD3 ganglioside mimic</td>
<td>Murine IgG2</td>
<td>Vaccine</td>
<td>SCLC</td>
<td>Phase III</td>
</tr>
<tr>
<td>CD 791</td>
<td>VEGF-2/KDR</td>
<td>Human di-Fab Linked with PEG Murine IgG1</td>
<td>Combination with chemotherapy</td>
<td>Melanoma</td>
<td>Phase II</td>
</tr>
<tr>
<td>CeaVac</td>
<td>Anti-idiotypic mAbs, CEA mimic</td>
<td>Human IgG2</td>
<td>Vaccine in combination with chemotherapy for CRC or TriAb for NSCLC None</td>
<td>PC Multiple Myeloma Metastatic esophagogastric cancer</td>
<td>Phase III Phase II</td>
</tr>
<tr>
<td>Denosumab</td>
<td>RANKL</td>
<td>Human IgG2</td>
<td>Combination with chemotherapy for advanced LC None</td>
<td>CRC</td>
<td>Phase III</td>
</tr>
<tr>
<td>EMD 72000 (Matuzumab)</td>
<td>EGFR</td>
<td>Humanized IgG1</td>
<td>Combination with chemotherapy</td>
<td>CRC</td>
<td>Phase III</td>
</tr>
<tr>
<td>HGS-ETR1 (Mapatumumab)</td>
<td>TRAIL-1</td>
<td>Human IgG1</td>
<td>None</td>
<td>NSCLC Advanced LC NHL</td>
<td>Phase II</td>
</tr>
<tr>
<td>HuMax-CD4 (Zanolimumab)</td>
<td>CD4</td>
<td>Human IgG1</td>
<td>None</td>
<td>T-Lymphoma FL</td>
<td>Phase III</td>
</tr>
<tr>
<td>HuMax-CD20 (Ofatumumab)</td>
<td>CD20</td>
<td>Human IgG1</td>
<td>Combination with chemotherapy for FL None</td>
<td>B-CLL</td>
<td>Phase III</td>
</tr>
<tr>
<td>HuMV833</td>
<td>VEGF-A</td>
<td>Humanized IgG4</td>
<td>Immunotoxin in combination with peptide vaccine for skin cancer</td>
<td>Advanced ovarian cancer and CRC CLL Skin cancer Melanoma Pancreatic cancer PC</td>
<td>Phase I Phase II</td>
</tr>
<tr>
<td>LMB-2 (α-TAC(Fv-PE38))</td>
<td>αCD 25</td>
<td>Human dsFv-PE38 fusion protein</td>
<td>Combined with vaccine or chemotherapy for melanoma</td>
<td>NHL</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>MDX-010 (Ipilimumab)</td>
<td>CTLA-4</td>
<td>Human IgG1</td>
<td>None</td>
<td>PC</td>
<td>Phase I</td>
</tr>
<tr>
<td>MDX-210</td>
<td>ErBb-2 X CD64 (FcγRI) ErBb-2</td>
<td>Murine F(ab')2 Bispecific</td>
<td></td>
<td>Ovarian cancer</td>
<td>Phase III</td>
</tr>
<tr>
<td>Omnitarg (Pertuzumab)</td>
<td>ErBb-2</td>
<td>Humanized IgG1</td>
<td>Combined with chemotherapy or with Trastuzumab</td>
<td>Breast cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>OVAREX (Oregovoma)</td>
<td>CA 125</td>
<td>Human IgG1</td>
<td>Combination with chemotherapy</td>
<td>Ovarian cancer</td>
<td>Phase II/III</td>
</tr>
<tr>
<td>PanoReX (Edrecolomab)</td>
<td>EpCam</td>
<td>Human IgG1</td>
<td>Combination with chemotherapy</td>
<td>Dukes’ C CRC</td>
<td>Approved in Germany 1995</td>
</tr>
<tr>
<td>Rencarex (WX-G250)</td>
<td>CA-IV+MAG250</td>
<td>Chimeric IgG1</td>
<td>90Yttrium for kidney cancer</td>
<td>Kidney cancer ARCC CLL NHL Meothelioma, ovarian, head and neck cancer</td>
<td>Phase I/II Phase I/II Phase I/II Phase I</td>
</tr>
<tr>
<td>SGN 40</td>
<td>CD40</td>
<td>Humanized IgG1</td>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SS1P (SS1 (dsFv)-PE38)</td>
<td>α-mesothelin</td>
<td>Human dsFv-PE38 fusion protein</td>
<td>Immunotoxin</td>
<td>Ovarian cancer</td>
<td>Phase II</td>
</tr>
<tr>
<td>Theragyn (Pemtumomab)</td>
<td>PEM</td>
<td>Humanized IgG1</td>
<td>90Yttrium for OC and GC</td>
<td>Ovarian cancer</td>
<td>Phase III</td>
</tr>
<tr>
<td>Zamyl CD33</td>
<td>Humanized IgG1</td>
<td>Vaccine in combination with surgery and chemotherapy for OC</td>
<td>Gastric cancer</td>
<td>Phase I – as vaccine Phase II</td>
<td></td>
</tr>
<tr>
<td>Zenapax (Daclizumab)</td>
<td>CD25</td>
<td>Humanized IgG1</td>
<td>Combination with chemotherapy</td>
<td>AML</td>
<td>Phase III</td>
</tr>
</tbody>
</table>

ALL, acute lymphocytic leukemia; AML, acute myelogenous leukemia; ARCC, advanced renal cell carcinoma; BC, breast cancer; CLL, chronic lymphocytic leukemia; CRC, colorectal cancer; GC, gastric cancer; FL, follicular lymphoma; NHL, non-Hodgkin’s lymphoma; NSCLC, non-small-cell lung cancer; LC, lung cancer; OC, ovarian cancer; PC, prostate cancer; PE38, Pseudomonas exotoxin A catalytic domain.
with ligand-activated ErbB-1/EGFR, ErbB-3 and ErbB-4 (Klapper et al., 1999). Consistent with this notion, the threedimensional structure of ErbB-2's ectodomain revealed an active conformation similar to that of ligand-activated EGFR (Garrett et al., 2003). In addition, ErbB-2null mice display phenotypes shared with both ErbB-3null (Erickson et al., 1997; Riethmacher et al., 1997) and ErbB-4null animals (Gassmann et al., 1995). Notably, ErbB-4null mice and ErbB-2null animals share a defect in cardiac muscle differentiation previously observed in animals lacking expression of a ligand for ErbB-4, neuregulin-1, an observation related to the major adverse clinical effect of Trastuzumab (see below).

A unique landmark of ErbB-2 is frequent overexpression in a variety of human adenocarcinomas (Slamon et al., 1989), primarily due to amplification of the corresponding portion of chromosome 17. A recent large compilation of studies confirmed the initial observation made by Dennis Slamon (Slamon et al., 1989), namely that ErbB-2 overexpression is associated with poor prognosis of breast cancer patients (Ross et al., 2003). Moreover, ErbB-2 expression is inversely correlated with steroid hormone receptors (Konecny et al., 2003). Breast cancer tumors that overexpress ErbB-2 are more likely to be of ductal rather than lobular origin, with higher grade, DNA aneuploidy, p53 mutations, and topoisomerase II amplification. In transgenic models, genomic amplification of ErbB-2 associates with tumor progression (Andrecheck and Muller, 2000), and multiple lines of in vitro evidence implicate overexpression with enhanced signaling due to retardation of growth factor dissociation, altered receptor trafficking and recruitment of the mitogen-activated protein kinase pathway, as well as the Akt/protein kinase B pathway (reviewed in Citri and Yarden (2006)). In summary, an overexpressed human ErbB-2, similar to a rodent mutant (Bargmann et al., 1986), harbors potent oncopgenic activities. Since high density of ErbB-2 molecules at the cell surface predicts response to immunotherapy (Mass, 2000), the mechanism of antibody action may entail inhibition of one or several oncogenic features of ErbB-2.

3. Clinical development of therapeutic antibodies

Rituximab (Rituxan, IDEC Pharmaceuticals, San Diego, CA, and Genentech Inc., San Francisco, CA) was originally approved for use against indolent B-cell non-Hodgkin’s lymphoma (NHL), but its use has expanded to other CD20-positive NHL, as well as to non-cancer disorders, such as autoimmune diseases. B-cell NHL corresponds to specific stages of normal B-cell development (Harris et al., 1994). Currently, NHL is one of the six most common malignancies in western societies and its current incidence not only increases with age, but also within any given age group. Rituximab has been approved based on a pivotal clinical trial demonstrating response rate of 48% in 166 indolent NHL patients (McLaughlin et al., 1998). The median duration of response was 11.6 months. Participants received an outpatient intravenous treatment course of the chimeric antibody (375 mg/m²) weekly for four weeks. Interestingly, in phase I trials, no maximum tolerated dose was achieved, due to lack of toxicity. Despite high efficacy and expression of CD20 in all patients treated with Rituximab, the antibody is active in only 50-60% of follicular lymphoma and 10-15% of small lymphocytic lymphoma (McLaughlin et al., 1998). Thus, similar to other antibody-treated diseases, there must exist mechanisms of antigen-independent resistance to therapy.

Trastuzumab (Herceptin, Genentech Inc., San Francisco, CA) is used at present as a single agent for patients with ErbB-2-overexpressing metastatic breast cancer, who received at least one regimen of chemotherapy. In addition, breast cancer patients whose tumors overexpress ErbB-2, and who have not received chemotherapy for their metastatic disease may be treated with a combination of chemotherapy (e.g., taxol) and Trastuzumab. Clinical testing of Trastuzumab followed observations of the antibody’s ability to inhibit proliferation of both cultured breast cancer cell lines, as well as tumorigenic growth of tumor xenografts (Hudziak et al., 1989; McKenzie et al., 1989). Early clinical trials demonstrated efficacy in the treatment of breast cancer patients whose ErbB-2-positive lesions relapsed following conventional treatment (Baselga et al., 1996; Pegram et al., 1998). A pivotal single arm phase II trial recruited patients who had progressive disease after one or two cytotoxic chemotherapy regimens for metastatic disease (Coleleigh et al., 1999). Patients (n = 220) received a loading dose of Trastuzumab (4 mg/kg) followed by weekly doses (2 mg/kg). Other than infusion-related symptoms, all other adverse effects were minor, with the exception of cardiac dysfunction in 10 patients. The overall response to Trastuzumab was 15% (Review Evaluation Committee) and 22% (investigator-assessed response). The median response duration and survival were 9.1 and 13 months, respectively.

A subsequent pivotal phase III combination trial tested safety and efficacy of a combination of Trastuzumab with chemotherapy versus chemotherapy alone (Slamon et al., 2001). Only ErbB-2-positive patients (n = 469), who had received no prior therapy for metastatic disease, were eligible for entry into the trial. Two chemotherapy regimens were used: anthracyclin plus cyclophosphamide (AC) or paclitaxel. The addition of Trastuzumab to AC increased the average time to progression (TTP) from 6.1 to 7.8 months, and the combination with paclitaxel was even more effective. Likewise, Trastuzumab combination therapy significantly improved other parameters, including median survival (25 months, compared with 20 months following chemotherapy alone). The most serious adverse event of Trastuzumab was an increased risk for cardiac dysfunction in patients receiving concomitant AC. Of particular interest is the fact that combination therapy was especially effective in the group of patients whose ErbB-2 expression was very high (3+ subset).

Antibody combination with chemotherapy is a shared feature of several therapeutic antibodies. This has been instigated with the observation that an antibody to EGFR can sensitize cancer cells to the cytotoxic effect of cisplatin (Aboud-Pirak et al., 1988), an observation later extended to other chemotherapeutic agents and irradiation, as well as to other antibodies, such as Trastuzumab. As a result, both clinically approved antibodies to EGFR, Cetuximab and Panitumumab (Vecitibix), are used in combination with chemotherapy to treat colorectal cancer. Further, the recently approved combination of Cetuximab and radiotherapy improves locoregional control and reduces mortality of patients with head and neck cancer (Bonner...
A phase II study of Rituximab in combination with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone) chemotherapy in patients with previously untreated, aggressive NHL and a median follow-up of 63 months, reported an overall response (OR) rate of 94% and complete response (CR) rate of 61% at the end of therapy (Vose et al., 2005). This durable effect of combination therapy on overall survival, and the absence of long-term adverse events, will be tested in ongoing randomized phase III trials. In vitro studies confirm the ability of Rituximab to chemoensitize several NHL B-cell lines (Bonavida and Vega, 2005). Similarly, various Trastuzumab combinations with chemotherapeutic agents revealed either synergistic interactions at clinically relevant drug concentrations, additive cytotoxic effects, or, rarely, antagonistic interactions (Pegram et al., 1999). As will be discussed below, the mechanism underlying the clinical benefit of combination therapy may not involve immune effector functions, but rather an intrinsic ability of several therapeutic antibodies to block survival pathways.

4. Mechanisms of action of therapeutic antibodies

Two major modes of action enable monoclonal antibodies to inhibit cancer cell growth: immune mechanisms and mechanisms that intercept pathways of tumorigenesis (see Figure 1). The immune mechanisms include antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), whereas mechanisms that intercept pathways of tumorigenesis include a large variety of machineries, such as triggering of apoptosis, blocking angiogenesis, inhibiting tumor cell proliferation, interfering with signaling cascades and accelerating receptor internalization.

ADCC is a well-recognized immune effector mechanism, in which antigen-specific antibodies direct immune effector cells (NK cells, monocyte-macrophages and leukocytes, especially neutrophils) to the killing of antigen-expressing cancer cells (Fannello and Ahmad, 2005). ADCC is a tripartite process involving three components: (i) the expression of the target antigen on cancer cells, (ii) the presence of the antigen-specific antibodies of the appropriate isotype, and (iii) Fc receptor-bearing effector cells. ADCC is associated with the anti-tumor cell effects induced by Rituximab, Trastuzumab, Daclizumab (anti-IL-2 receptor; CD25) and other antibodies (Clynes et al., 2000; Ravetch and Lanier, 2000). Murine xenogenic models of human tumors were shown to involve the association of the antibody with Fc receptors (FcRs) type I, II, or IV (Nimmerjahn et al., 2005). Significant diminished efficacy of antibodies directed toward malignant cells was demonstrated using FcRγ−/− deficient mice that do not express FcγRIII, the stimulatory Fc receptor. In contrast, mice deficient in the ADCC inhibitory FcγRIIB are hypersensitive to antibody-mediated tumor suppression. Clinical trials correlating the response of NHL patients to Rituximab with polymorphisms of Fc receptors support a role for ADCC in immunotherapy (Cartron et al., 2002; Weng and Levy, 2003). Studies involving FcγRI-deficient mice (Clynes et al., 2000), as well as bivalent fragments of anti-ErbB-2 mAbs lacking the Fc portion (Spiridon et al., 2004), have shown that ADCC is a predominant mechanism of action of anti-ErbB-2 antibodies.

Some observations suggest that the outcome of patients who receive Rituximab can be improved if the amount and function of their immune effector cell population (in

![Figure 1 – Mechanisms of action of therapeutic antibodies. Binding of mAbs (orange) to antigens (green) on target cells can induce complement binding (via the C1 component) and activate ADCC, which requires interaction between the Fc portion of the antibody and FcγR molecules on effector cells, such as natural killer (NK) lymphocytes. Angiogenesis and cell growth are inhibited through downregulation of the vascular endothelial growth factor (VEGF) and mAbs interfering with growth factor (GF) binding, respectively. Intracellular degradation of surface antigens is preceded by endocytosis (internalization) of antigen-bound mAbs (e.g., Trastuzumab). In the case of Rituximab, apoptosis occurs upon tyrosine kinase signaling and mobilization of intracellular calcium.](image)
particular, NK cells) are preserved or enhanced. Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a cytokine that strongly increases the number and activity of polymorphonuclears and macrophages against opsonized targets (Bober et al., 1995). Therefore, attempts to improve Rituximab efficacy are directed toward combined treatments with GM-CSF, based upon the evidence that this cytokine can up-regulate CD20 expression on lymphoid B cells and enhance ADCC (Olivieri et al., 2005). Similarly, it has been suggested that IL-2 can promote NK cell development and enhance Rituximab activity (Olivieri et al., 2005). Alternative approaches such as enhanced effector cell activity induced by CpG oligonucleotides are also under investigation (Lopes de Menezes et al., 2007).

6. Complement-dependent cytotoxicity

Complement lysis is controlled both by a series of zymogen activators (C1–C9) and by a series of inhibitory proteins (e.g., CD35, CD46, CD55 and CD59). Direct evidence for the role of CDC is reflected by the reduced efficacy of Rituximab in animals lacking C1q, the first component of the classical complement pathway (Di Gaetano et al., 2003). Likewise, complement inhibitory proteins have been shown to inhibit Rituximab-mediated cell killing, and blocking the inhibitors enhanced Rituximab-mediated CDC (Golay et al., 2001). Similarly, Trastuzumab was shown to mediate CDC in vitro when human immune effector cells and sera were used, but it was ineffective when the immune effector cells and sera were of mouse origin (Spiridon et al., 2002). Another line of evidence supporting CDC involvement is reflected by the ability of β-glucan to enhance the effects of Rituximab in animals (Modak et al., 2005). The enhancement effect is mediated by the CR3 receptor molecule of granulocytes, which recognizes tumor cells coated with the inhibitory iC3b component of the complex (Hong et al., 2004). Taken together, CDC and ADCC are thought to be important effector mechanisms of therapeutic monoclonal antibodies. Moreover, it was suggested that CDC induced by Rituximab depends on CD20 density, and that CDC and ADCC act complementarily (van Meerten et al., 2006).

7. Interception of oncogenic pathways

7.1. Enhancing cancer cell apoptosis

Apoptosis, or programmed cell death, occurs through two main pathways: the extrinsic pathway can be triggered by cytokines (e.g., TNF and TRAIL) and the intrinsic (or mitochondrial) pathway, leading to cytochrome release from mitochondria and downregulation of anti-apoptotic Bcl-2 family proteins. Both pathways converge to activate a cascade of proteases, caspases, which cleave regulatory and structural molecules. Several therapeutic antibodies have been implicated in the induction of apoptosis via the intrinsic pathway. In the case of Rituximab, apoptosis is induced by two main alleys: either via accelerating calcium flux, or by direct induction of the intrinsic pathway. CD20 can act as a calcium channel modulator (Bubien et al., 1993). In addition, crosslinking of CD20 molecules stimulates certain Src-family protein tyrosine kinases, which activate a phospholipase, mobilize calcium ions and activate caspase-3 (Hofmeister et al., 2000). Similar to Rituximab, Trastuzumab induces apoptotic pathways within ErbB-2-expressing mammary tumors (Mohsin et al., 2005). The pro-apoptotic activity of Trastuzumab has been attributed to inhibition of constitutively activated MAP-kinase and Akt pathways downstream to ErbB-2, and to TRAIL-induced apoptosis (Cuello et al., 2001). In the case of EGFR, it has been reported that treatment with Cetuximab or with similar anti-EGFR antibodies elevated expression of Bax (pro-apoptosis) and decreased Bcl-2 (anti-apoptosis) (Huang et al., 1999).

7.2. Inhibiting tumor cell proliferation

Cell cycle progression is controlled by a series of cyclin-dependent kinases (CDKs), their activators (Cyclins), or inhibitors. For example, elevated expression of p27Kip, a key regulator of the G1/S transition, arrests cells at the G1/S phase. Rituximab in combination with irradiation up-regulates expression of p27Kip and inhibits cell growth (Skvortsova et al., 2006). It was proposed that Rituximab-induced growth inhibition is mediated through a ceramide-triggered signaling pathway, leading to the induction of p27Kip through a MAP-kinase-dependent mechanism (Bezombes et al., 2004). As in the case of Rituximab, cells treated with Trastuzumab undergo arrest during the G1 phase of the cell cycle, accompanied by reduced expression of proteins involved in sequestration of p27Kip, including Cyclin D1. This results in the release of p27Kip, allowing it to bind and inhibit Cyclin E/CDK2 complexes (Lane et al., 2001). Trastuzumab treatment also results in an accumulation of p27Kip, although this seems to occur secondary to the formation of p27Kip–CDK2 complexes, perhaps as a result of decreased targeting of p27Kip to the ubiquitin-proteasome. Other antibodies, such as Cetuximab and M225 (its murine progenitor), also interfere with cell cycle progression through the involvement of p27Kip in G1 arrest (Peng et al., 1996).

7.3. Curtailing angiogenesis

Tumor cells must use the process of vascularization, or angiogenesis, for productive growth and metastasis (Saaristo et al., 2000). The formation of a vascular network in tumors involves several angiogenic factors, including the vascular endothelial growth factor (VEGF-A), which promotes endothelial cell survival and proliferation. Transcription of VEGF mRNA is induced by low oxygen conditions (hypoxia), as well as by different growth factors and cytokines, including EGF family members. Attempts aimed at blocking angiogenesis by targeting either VEGF or its major receptor, VEGFR-2, yielded a murine antibody, which targets all isoforms of VEGF-A. The humanized antibody not only inhibited growth of endothelial cells, but also retarded tumor growth in animals (Presta et al., 1997). Moreover, when combined with chemotherapy or radiotherapy, the humanized antibody, Bevacizumab (Avastin, Genetech/Roche), enhanced tumor suppression in animals (reviewed in Ferrara (2005)). These preclinical results, along with mild and reversible adverse effects observed in
monkeys, formed the foundation for an extensive program of clinical trials, which led to the approval of Bevacizumab in combination with chemotherapy for the treatment of metastatic colorectal cancer, and many ongoing tests in other malignancies. An alternative strategy targets the receptor for VEGF, VEGFR-2 (Lu et al., 2003). The high-affinity antibody prevents the binding of VEGF, and prolongs survival of animals grafted with human leukemic cells. Interestingly, when tested in mice Trastuzumab induced normalization and regression of the vasculature in an experimental human breast tumor (Izumi et al., 2002). The mechanism may involve downregulation of VEGF production by tumor cells (Petit et al., 1997). Furthermore, according to in vitro lines of evidence, Trastuzumab better inhibits angiogenesis when combined with chemotherapy (e.g., paclitaxel).

### 7.4. Interfering with signaling cascades

Therapeutic antibodies may activate cellular signaling pathways culminating in retardation of malignancy. Binding of CD20 by Rituximab, especially if cross-linked, activates a phosphatidylinositol- (PI-) specific phospholipase (PLC-γ) leading to p38-MAP-kinase activation, and apoptosis which can be inhibited by a p38-specific inhibitor (Pedersen et al., 2002). PLC-γ generates a second messenger (IP₃) which leads to calcium flux-mediated apoptosis (Hofmeister et al., 2000). CD20 density and clustering by Rituximab determine the extent of response, in part because ligated molecules rapidly redistribute into lipid rafts (Deans et al., 2002). Interestingly, anti-EGFR and anti-ErbB-2 mAbs often induce tyrosine phosphorylation by dimerizing the respective receptor (Yarden, 1990), but only rarely do they enhance cell growth (Stancovski et al., 1991). According to one explanation, antibodies may stimulate inhibitory pathways, such as the PTEN phosphatase. PTEN (MMAC1/TP) dephosphorylates position D3 of membrane phosphatidylinositol-3,4,5-triphosphate (PI(3,4,5)P₃), thereby dissociating AKT from the cell membrane and negatively regulating the enzyme (Sulis and Parsons, 2003). PTEN expression is reduced following activation of cells with ErbB ligands (Nicholson et al., 2003), but Trastuzumab treatment rapidly increases membrane localization and phosphatase activity, by reducing PTEN tyrosine phosphorylation via Src inhibition (Nagata et al., 2004). Moreover, a decrease in PTEN expression confers resistance to Trastuzumab. Another potential mechanism of Trastuzumab action relates to a slow extracellular proteolytic cleavage of ErbB-2, yielding a p100-ErbB-2 (extracellular) and a p95-ErbB-2 (cytoplasmic domain), which appears to be a constitutively active kinase (Christianson et al., 1998). Because Trastuzumab inhibits ErbB-2 cleavage by surface metalloproteinases (Molina et al., 2001), diminished production of the active p95 fragment may underlie the inhibitory effect of the therapeutic antibody.

### 7.5. Accelerating receptor internalization

Ligand-induced ubiquitinylation and internalization of EGFR and other receptor tyrosine kinases are considered a major cellular desensitization processes (Marmor and Yarden, 2004). In this vein, preventing membrane localization of ErbB-2 abolishes its transforming potential (Flanagan and Leder, 1988; Beerli et al., 1994). Unlike growth factors, antibodies to EGFR and ErbB-2 induce relatively slow internalization, but both routings converge at a pre-lysosomal compartment (Maier et al., 1991). According to an alternative model, the relatively slow antibody-induced degradation of ErbB-2 is due to dynamic recycling of Trastuzumab-receptor complexes (Austin et al., 2004). Whether or not antibody-induced internalization and degradation of oncogenic receptors contribute to the therapeutic potential of anti-ErbB proteins remains an open issue. Analysis of two internalizing monovalent single chain antibody fragments, which exerted no sustained growth inhibitory effects, implied that antibody-induced internalization of ErbB-2 may not play a role in growth arrest (Neve et al., 2001). On the other hand, comparison of tumor-inhibitory anti-ErbB-2 antibodies and a tumor-stimulatory antibody revealed that the former, unlike the latter, accelerated degradation of both ErbB-2 and the antibody (Hurwitz et al., 1995). Moreover, combining two antibodies that bind to distinct determinants of either EGFR or ErbB-2 significantly accelerated receptor degradation (Friedman et al., 2005), as well as enhanced ADCC and tumor inhibition (Spirodon et al., 2002). Likewise, continuous exposure of cells to Trastuzumab results in downregulation of the cell surface ErbB-2, a concurrent increase in the level of p27kip1 and arrest of cell cycle progression (Marches and Uhr, 2004). Furthermore, according to a recent study, resistance to Trastuzumab may be attributed to autocrine secretion of EGFR ligands, which inhibit antibody-induced endocytosis of ErbB-2 (Valabrega et al., 2005). Unlike receptor tyrosine kinases, internalization may not contribute to the therapeutic potential of Rituximab. CD20 is considered an ineffectively internalizing antigen, although it follows the canonical clathrin-mediated pathway (Pulczynski et al., 1994), and reaches the recycling endosomal compartment (Pulczynski et al., 1994). Nevertheless, the ability of antibody-crosslinked CD20 to deliver cytotoxic drugs to cancer cells greatly enhances the effects of Rituximab, especially when the drug is designed to be released in lysosomes (DiJoseph et al., 2006).

### 7.6. Antagonizing ligand binding to tumor cells

Binding of EGF and other growth factors instigates a plethora of signaling events culminating in cell proliferation or migration. Both Cetuximab and Panitumumab block ligand binding to EGFR, and consequently, inhibit receptor autophosphorylation (Gill et al., 1984; Yang et al., 1999). Nevertheless, experiments performed in animals indicate that the anti-tumor effects of antibodies to EGFR are independent of the ability to antagonize ligand binding (Mendelsohn and Baselga, 2000). Like EGFR, CD22, an adhesion receptor for sialic acid-bearing ligands, interacts with a number of kinases and phosphatases that bind the cytoplasmic domain through phosphorylated tyrosine residues. A ligand-blocking anti-human CD22 antibody exhibits increased cytotoxic effects on human CD22⁺ tumor cells, compared with non-blocking mAbs (Tuscano et al., 2003). Nevertheless, Pratuzumab, a humanized anti-CD22 monoclonal antibody, currently in clinical trials for treatment of NHL (Carnahan et al., 2007), binds to a region outside of the two amino-terminal ligand-binding domains of CD22 (Stein et al., 1993).
8. At last, adjuvant antibodies are entering clinical application

Isolated tumor cells in the bone marrow or in other tissues, often remote from the primary lesion, may be regarded as the precursors of potentially fatal distant metastases. For example, approximately one third of node-negative breast cancer patients develop local or distant metastases, despite undetectable tumor spread at diagnosis (Bonner et al., 2006). The ability of therapeutic antibodies, such as Trastuzumab and Cetuximab, to detect antigen-overexpressing tumor cells independent of cell cluster’s size, makes them suitable for micrometastases targeting. In addition, because clinical management of micrometastases requires long-term treatment, the relatively mild adverse effects of most therapeutic monoclonal antibodies make them ideal for adjuvant therapy. In line with this rationale, evidence from cross-trial analysis suggests that the clinical benefits of Trastuzumab are greater if the antibody is administered earlier in the course of the disease (Bell, 2002). Motivated by these considerations, an urgent clinical need, and the ability of Trastuzumab to extend survival of HER2-positive metastatic breast cancer patients, several large multicenter trials, including in between them more than 13,000 patients, were designed to test Trastuzumab as adjuvant therapy after surgical treatment of primary breast cancer (Baselga et al., 2006).

Notwithstanding differences in patient populations, sequencing of treatments and chemotherapy regimens, and despite the relatively short (1–2.5 years) follow-up time, adjuvant therapy with Trastuzumab has consistently shown a significant reduction in the risk of early relapse. The reported magnitudes of reduction and the overall consistency of results are considered remarkable, and Trastuzumab is rapidly becoming the standard care after surgery for high-risk, ErbB-2-positive cancer. Since the clinical lessons learned in the adjuvant Trastuzumab trials are relevant to future development of therapeutic antibodies targeted at other antigens, we concisely review below the critical efficacy and safety data. Two North American randomized trials (NASBP B31 and Intergroup N9831) tested the standard chemotherapy regimen of doxorubicin plus cyclophosphamide (AC), followed by paclitaxel and 1 year with or without concurrent Trastuzumab (Romond et al., 2005). It is notable that no standard regimen for the treatment of women with node-positive breast cancer has been adopted in Europe or elsewhere, outside the United States. The HERA international trial (non-US) recruited 5090 patients who have completed at least four cycles of adjuvant therapy, and then treated (or untreated) with Trastuzumab for 1 or 2 years (Piccart-Gebhart et al., 2005). The fourth large international trial (BCIRG006), a three-arm study, treated with docetaxel plus carboplatin and Trastuzumab, or with AC followed by docetaxel, in comparison with AC followed by docetaxel and Trastuzumab (Slamon et al., 2005). A relatively small Finnish study randomized 232 patients into two arms of treatment: docetaxel or vinorelbine followed by fluorouracil, epirubicin and cyclophosphamide, which were combined with Trastuzumab in one arm of the study (Joensuu et al., 2006).

Taken together, the large adjuvant trials have shown that Trastuzumab reduces the 3-year risk of breast cancer recurrence by approximately 50%. For example, the combined analysis of trials NSABP B-31 and N9831 indicates that Trastuzumab significantly prolongs disease free survival time, with an absolute difference of 12% between groups at 3 years. The corresponding benefit indicated by HERA at 2-years was 8.4%. Notably, the design of HERA is unique as it includes Trastuzumab monotherapy administered sequentially (rather than concomitantly to chemotherapy), a flexible approach to previous chemotherapy (only one third of patients received previous taxanes) and recruitment of both node-positive and node-negative patients. With longer follow-up, the comparison of HERA’s disease free survival curves with those of other trials will likely highlight the power of taxanes and the significance of administration schedule. Despite differences, it is remarkable that in the adjuvant setting Trastuzumab abrogated the commonly observed initial peak of recurrences occurring in the first 2–3 years. Whether or not this effect is due to antibody- or complement- mediated cytotoxicity of micrometastases remains an open question.

Although long-term treatment with Trastuzumab in the adjuvant setting emerges as an acceptable treatment from a safety perspective, there have been differences among the trials. The docetaxel–carboplatin–Trastuzumab arm of BCIRG 006 was associated with treatment-related death (0.8%), and 7% of patients of the Trastuzumab arm of HERA experienced mild or serious effects (4.7% in the control arm). The incidence of cardiac events with Trastuzumab, 0.6–3.3% higher than the respective control arm, was similar across the adjuvant trials. Nevertheless, the majority of patients who experienced treatment-related congestive heart failure improved upon treatment. Thus, adjuvant Trastuzumab is well tolerated and the risk-benefit ratio demonstrated in the clinical trials provide compelling reasons to consider adjuvant antibody treatment for women with ErbB-2-positive mammary tumors.

9. Future directions

The packed full pipeline of monoclonal antibodies currently undergoing oncological trials promises a steady ascending trajectory, which will likely surpass the rate witnessed in the first decade of clinical application. While it is difficult to predict the scope of future adjuvant antibody therapy, analogous to the newly approved use of Trastuzumab, it seems safe to argue that the antibody–cytotoxics therapeutic alliance is here to stay. The combination of antibodies and chemo- or radiotherapy poses a major challenge for basic researchers, since the underlying basis of cooperation remains largely unknown. The reward, however, is luring: once resolved and established at the level of predictive molecular markers, the mechanism of synergy may enable optimization of drug combination, as well as guide the selection of appropriate doses and schedules. Similarly, more efficacious cancer-inhibitory antibodies will likely follow improvements in our understanding of how certain antibodies inhibit cancer cells in vivo. Engineered immune effector functions, as well as enhanced anti-tumor intrinsic activities, will likely emerge from better mechanistic understanding. Another expected outcome is
more robust and cost-effective expression systems, which will help reduce the current high costs of antibody therapy.

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