CHAPTER 1

ANTIBODY-MEDIATED DRUG DELIVERY SYSTEMS: GENERAL REVIEW AND APPLICATIONS

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1 HISTORICAL PERSPECTIVE

The term *antibody* was first used by Paul Ehrlich in year 1891 in his article "Experimental Studies on Immunity." In 1890, Emil Von Behring and Shibasaburo Kitasato established the basis for *serum therapy*: that serum taken from animals treated with nonlethal doses of diphtheria and tetanus can be used for the treatment of diphtheria and tetanus. They followed this discovery with the *theory of humoral immunity*, which prompted Paul Ehrlich to propose *side chain theory*, which describes the interaction between antibodies and antigens. Later, in the 1920s and the 1930s, it was shown by Michael Heidelberger and Oswald Avery that antibodies are made of protein, and the biochemical aspect of antigen–antibody interactions was explained by John Marrack. In the following years, the structure of antibodies was characterized by a number of scientists independently [1].

In 1975, Köhler and Milstein successfully produced antibodies in vitro using "hybridoma technology." This discovery allowed the production and use of antibodies on a large scale for diagnostic and therapeutic purposes. The first

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antibody, OKT3, was approved by the U.S. Food and Drug Administration (FDA) in 1986 for use in patients to prevent transplant rejections [2]. Since then, numerous technologies have been developed to decrease the immunogenicity of mouse antibodies by generating partial or fully human antibodies. A total of 28 therapeutic antibodies approved by the FDA are currently available in the U.S. market. It is the fastest-growing market, and its revenue is expected to increase to $62.7 billion in 2015, according to DatamonitorPlc, a London-based health information firm [3].

2 ANTIBODIES

Antibodies (also known as *immunoglobulins*) are proteinacious in nature and are produced in response to an invasion of foreign substances in the body called *antigens*.

2.1 Structure of Antibodies

Antibodies are heavy (~150 kDa), Y-shaped glycoproteins composed of four polypeptide chains: two long *heavy* or *H chains* and two short *light* or *L chains*. The end of light and heavy chains together constitutes a variable region (also known as *antigen-binding site*) consisting of 110 to 130 amino acids. The amino acid sequence in the variable region gives antibody its specificity for binding to a variety of antigens.

2.2 Types of Antibodies

There are five major types of antibodies, each having a specific role in the immune response:

1. *IgG*: comprises 75 to 80% of total antibodies circulating in the blood and body fluids. This is the principal antibody found in the body and provides the majority of antibody-mediated protection against bacterial and viral infections. It is produced one month following initial B-cell activation.
2. *IgA*: comprises 10 to 15% of total antibodies present in the body. These are involved predominantly in the protection of mucosal surfaces exposed to various pathogens and are thus found in mucosal areas such as the digestive tract, the respiratory tract, the urogenital tract, and the eyes.
3. *IgM*: makes up about 5 to 10% of total circulating antibodies in the body. IgM antibodies are the first to appear in the body post-infection. They are expressed on the surface of B cells and are also secreted by them.
4. *IgD*: comprises about 1% of total antibodies present in the body. The exact function of IgD antibodies is not very clear.
5. *IgE*: makes up about 0.05% of all immunoglobulins in the body. IgE binds to Fc receptors on the surface of mast cells and basophils to produce an immune response. These are particularly involved in allergic reactions and immune responses to parasitic worms [4–7].

2.3 Antibody Development

Over a period of time, numerous methods have been devised for the production of antibodies, the first being the *hybridoma method* proposed by Köhler and Milstein. This method involves immunization of mice with a mixture of antigens followed by fusion of their spleen cells with immortalized myeloma cells. These cells are then cloned and screened for production of the desired antibodies. Certain limitations associated with the method involve specificity issues, as the antibodies are derived from murine cells and thus resemble a rodent immune system and also because these antibodies are recognized as allogenic proteins in human patients, which leads to human antimouse antibody response.

Another method, the *Epstein–Barr virus method*, involves immortalization of human cells by the Epstein–Barr virus. The disadvantage of this method is its nonspecificity in terms of immortalizing antigen-specific B cells among a pool of peripheral blood lymphocytes.

To humanize murine antibodies further, chemical and molecular methods were devised, such as replacement of the Fc portion of murine antibodies by that of human antibodies to yield chimeric monoclonal antibodies. Also, immortalization of genes corresponding to specific antibodies, and grafting of DNA fragments determining the binding specificity of the antibody into the framework of human immunoglobulin genes, leads to the production of humanized antibodies.

The *phage display method* is an efficient method for the production of high-affinity antibodies. It involves ligation of a DNA library derived from B cells onto a surface protein gene of a bacteriophage. Further, phages expressing the required specificities are isolated, enriched, and used to infect *Escherichia coli* for the production of monoclonal antibody construct [8].

3 ANTIBODY MEDIATION

Antibody-mediated immunity is also called *humoral immunity* or *humoral immune response*. Lymphocytes (white blood cells) are divided into two types: *B lymphocytes* or *B cells* (which secrete antibodies and are involved in humoral immunity) and *T lymphocytes* or *T cells* (which are involved in cell-mediated immunity). Both types of cells originate from the bone marrow; they become B or T cells depending on their point of maturation. T cells develop in the thymus gland; B cells develop in the bone marrow. Antibodies are produced in the body by B lymphocytes or B cells. B cells develop in the bone marrow and travel from bone marrow to the spleen. Once in the spleen, the B cells undergo a maturation process during which the genes responsible for generating antibody recombine several times. This process renders the cells highly specific for a single antigenic sequence. During maturation, each B cell undergoes selection mechanisms which ensure that it is not only specific for one antigen, but also that it does not recognize self-antigen. During this process, any B cells that recognize self-antigen either die or their activity is permanently suppressed. When a B cell has gone through the entire recombination process, it becomes fully mature. Once fully matured, the cell is at a stage where it

will activate only when it recognizes a particular amino acid sequence during the course of a pathogenic infection. Mature B cells circulate throughout the body, via the bloodstream and lymphatic system, until they come into contact with the specific antigen that they recognize. When there is an infection, the invading pathogen produces antigen. Resting or naive B cells get activated when the antigen binds to its membrane, and this results in the production of numerous antibodies that bind specifically to that antigen. B cells can be activated in a T-cell-dependent or T-cell-independent manner.

1. *T-cell-dependent activation*. In this process, the B cells get help from T cells in the antibody response by acting as antigen-specific antigen-presenting cells. Ig receptors on the membrane of B cells bind antigens and internalize them by means of receptor-mediated endocytosis (a process by which cells absorb molecules such as proteins by engulfing them in vesicles). The pathogen is then digested in endosomal vesicles to yield peptide fragments, which are then attached to class II (major histocompatibility complex (MHC)) proteins and migrated to the plasma membrane of the B cells. Helper T cells recognize MHC–peptide complex on the surface of B cells and get stimulated to produce cytokines, which leads to activation and proliferation of B cells. Activated B cells subsequently mature into antibody-producing plasma cells which produce antibodies specific for the antigen presented to fight the infection. Once these antibodies are released into the bloodstream, they lock onto specific antigen. These antibody–antigen complexes are removed through the complement system or by the liver and spleen [9].

2. *T-cell-independent activation*. This process involves stimulation of antibody production in the absence of helper T cells. Many antigens are T-cell-independent and can deliver the signals directly to the B cell. T-cell-independent activation is brought about by T-cell-independent antigens such as polysaccharides, glycolipids, and nucleic acids. These antigens are not processed and presented along with MHC proteins and hence cannot be recognized by helper T cells. Many bacteria have repeating carbohydrate epitopes. Most of these antigens have multiple identical epitopes, which induces cross-linking of Ig receptors on B-cell surfaces and further stimulation of B cells, and there is no requirement for participation by antigen-specific helper T cells. These T-cell-independent (TI) antigens are of two types: *TI-1 antigen* is made up of lipopolysaccharide (LPS), and *TI-2 antigens* are polysaccharides, glycolipids, and nucleic acids. TI-1 antigens stimulate the B cells directly without the requirement of any other cell. At lower concentrations, gram-negative bacterial LPS stimulates specific antibody production, but at higher levels it acts as a polyclonal B-cell activator, stimulating growth and differentiation of most of the B cells without binding to the membrane receptors [10–12].

4 ANTIBODY-MEDIATED DRUG DELIVERY SYSTEMS

1. *Radioimmunotherapy*: a treatment method that employs radionuclide-labeled antibody to deliver cytotoxic radiation to target cells. Owing to the specificity of antibodies for the cancer antigens, radiolabeled antibodies have the

ability to localize in cancer cells and to kill the cells because of the cytotoxic radiations of radionuclide. Radioimmunotherapy has advantages over traditional chemotherapy, which distributes drug throughout the body (lack of selectivity) and is often associated with dose-limiting toxicities to various organs, and also over conventional radiation therapy, which has the disadvantage of killing normal healthy cells in addition to cancer cells. In addition to these advantages, radioimmunotherapy is better than conventional immunotherapy, as radiolabeled antibodies not only kill the cells to which they are bound but also the adjoining cancer cells [13].

Immunomedics, Inc. and IBC Pharmaceuticals, Inc. have designed a bispecific antibody, TF2, using patented dock-and-lock (DNL) protein engineering platform technology for pretargeted radiation therapy. Radiolabeled TF2 binds to carcinoembryonic antigen (CEA) and accumulates in CEA-expressing tumors, resulting in increased signal at tumor relative to nontumor tissues. Radiation can be targeted specifically to tissues bearing tumors. Results from the preclinical study of TF2 for pretargeted therapy suggests a fivefold increase in survival in one model and a twofold increase in another model. Temporary and mild side effects were found to be bone marrow and kidney toxicity. It is currently in early phase I studies with colorectal cancer [14].

2. *Immunoliposomes*: liposomal formulations with an encapsulated active agent and conjugated antibodies and antibody fragments on their surfaces. Antibodies and antibody fragments specific for certain tumor markers can be used for the targeted delivery of liposomes and can also help in internalization, owing to their ability to endocytose, resulting in overall improved bioavailability of chemotherapeutic agents. Various internalizing single-chain variable fragment (scFv) antibody fragments have been identified and are being used to deliver drugs to cancer cells, such as anti-CD166 scFv and a novel UA20 scFv which targets prostate cancer cells; anti-ErbB2 F5 scFv, which binds specifically to ErbB2 expressed on certain tumors; and anti-epidermal growth factor receptor (EGFR) scFv antibodies, which target EGFR overexpressed in a number of cancer cells [15,16]. Immunoliposomes have enhanced performance compared to liposomes, as these can be specifically targeted and internalized in cancer cells [17].

3. *Immunotoxins*: conjugates of antibody fragments linked chemically or genetically to toxins derived from bacterial, plant, or animal sources. Various toxins, such as *Pseudomonas*, anthrax and diphtheria (bacterial toxins), ricin, saporin, abrin, gelonin and pokeweed (plant toxins), restrictocin (fungal toxin), and hemolytic toxin from sea anemone (animal toxin), are being used for the treatment of cancer.

Denileukindifitox (Ontak) is an FDA-approved immunotoxin used for the treatment of cutaneous T-cell lymphoma. It is composed of interleukin-2 (IL-2) protein sequences conjugated to diphtheria toxin. IL-2 moiety of Ontak targets tumor cells expressing IL-2 receptors and delivers the immunotoxin inside the cells via receptor-mediated endocytosis, where diphtheria toxin fragment A is released into the cytosol, inhibiting the protein synthesis through the ADP ribosylation

FIGURE 1 Schematic representation of an antibody–drug conjugate.

of elongation factor 2 and leading to cell death [18]. Several immunotoxins are currently under development and in clinical trials.

A new anti-fAChR (fetal acetylcholine receptor) immunotoxin (scFv35-ETA) is currently being developed for the treatment of rhabdomyosarcoma (RMS). It is composed of fully human anti-fAChR Fab fragment fused to *Pseudomonas* exotoxin A. It showed promising results in vitro (killed RMS cell lines TE-671, FL-OH-1, and RD in a dose-dependent manner) and delayed RMS development in a murine transplantation model [19].

4. *Antibody–drug conjugates*: monoclonal antibodies linked or conjugated to cytotoxic drugs by means of a chemical linker. Antibody–drug conjugates exert their therapeutic efficacy by targeting the cytotoxic agents to tumors as a result of the ability of antibodies to recognize and bind specifically to tumor-specific and/or overexpressed antigens on cancer cells. Antibody–drug conjugates are superior to treatment with either monoclonal antibodies alone or cytotoxic drugs. Monoclonal antibodies can be used as single agents for the treatment of cancer; however, their efficacy is limited. Also, the efficacy of chemotherapy is limited because of lack of selectivity of cytotoxic agents, which leads to nonspecific toxicity of healthy tissues. In antibody–drug conjugates, antibody is attached to a cytotoxic drug by means of a linker (Fig. 1).

The challenges associated with antibody–drug conjugates are that the linker in these conjugates must be stable while circulating in the bloodstream and must release the drug while inside the tumor cells. Also, the conjugation must not affect the binding specificity of the antibody toward antigen and must be internalized effectively inside the cancer cells to attain sufficient intracellular drug concentration so as to kill the tumor cells [20,21]. Numerous antibody–drug conjugates currently on the market and under development are listed in Table 1.

5 APPLICATIONS

1. *Diabodies*. Diabodies are medium-sized bivalent and bispecific antibody fragments with a molecular weight of about 60 kDa. Diabodies consist of variable domains of heavy and light chains connected by a peptide linker. The short linker between the heavy and light domains hinders pairing between them while promoting pairing with the complementary domains of another chain, resulting in the formation of dimers called diabodies. Diabodies bind to multimeric antigens with great avidity because of their bivalency, and this leads to high tumor retention. Because of such advantages as rapid tissue penetration, high target retention, and rapid blood clearance, diabodies are particularly suitable for such applications as radioimmunotherapy and imaging.

TABLE 1 Antibody–Drug Conjugates Under Development

Agent	Target	Indication	Clinical Status
SAR566658	CA6	Breast, ovarian, cervical, lung, and pancreatic tumors	Phase I [22]
SAR650984	CD38	Hematological malignancies	Phase I [22]
BIIB015	Cripto	Solid tumors	Phase I [22]
BT-062	CD138	Multiple myeloma	Phase I [22]
Milatuzumab	CD74	Multiple myeloma, non-Hodgkin lymphoma, and chronic lymphocytic leukemia	Phase I/II [23]
Clivatuzumab	Mucin	Pancreatic cancer	Phase Ib [23]
Veltuzumab	CD20	Non-Hodgkin lymphoma and autoimmune diseases	Phase I/II [23]
Epratuzumab	CD22	Non-Hodgkin lymphoma and autoimmune diseases	Phase III [23]
AGS-16M18	AGS-16	Kidney and liver cancer	Phase I [24]
SGN-75	CD70	Non-Hodgkin lymphoma and renal cell carcinoma	Phase I [25]
Brentuximab vedotin	CD30	Hodgkin lymphoma	Phase III [26]
ASG-5ME	SLC44A4 (AGS-5)	Epithelial tumors	Phase I [27]
Dacetuzumab	CD40	Non-Hodgkin lymphoma and multiple myeloma	Phase Ib–IIb [28]
SGN-70	CD70	Autoimmune and inflammatory disorders	Phase I [29]
SGN-19A	CD19	Non-Hodgkin lymphoma, chronic lymphocytic leukemia, and acute lymphoblastic leukemia	Preclinical phase [30]
IMGN388	α_v integrin	Solid tumors	Phase I [31]
SAR3419	CD19	Non-Hodgkin lymphoma	Phase II [32]
Lorvotuzumab mertansine (IMGN901)	CD56	Solid tumors	Phase I/II [33]
Trastuzumab-DM1 (T-DM1)	HER2	Breast cancer	Phase III [34]
CDX-011	Glycoprotein NMB (GPNMB)	Breast cancer	Phase IIb [35]
CDX-014	TIM-1	Ovarian and renal cancer	Preclinical phase [35]
Anti-CD22	CD22	Hematologic malignancies	Phase I [36]
Vicinium and proxinium (VB4-845)	EpCAM	Bladder, head, and neck cancer	Phase II [37]

C6.5 diabody, a noncovalent anti-HER2 single-chain Fv dimer labeled with astatine-211 (^{211}At), injected in immunodeficient nude mice bearing established HER2/*neu*-positive tumors, resulted in 60% tumor-free animals after one year [38].

The potential of anti-EMP2 diabodies for the treatment of endometrial cancer was established by the results of in vitro and in vivo studies. In vitro treatment of endometrial adenocarcinoma cells with anti-EMP2 diabodies resulted in significant decreased cell proliferation by up-regulating caspase-dependent apoptosis and led to decreased tumor size and induced cell death in human endometrial cancer xenografts [39].

2. *Nanobodies*. Nanobodies, proteinaceous fragments derived from antibodies having a single variable domain, are also called *domain antibodies* (dAbs) or *single-domain antibodies* (sdAbs). Nanobodies (12 to 15 kDa) are much smaller than the whole antibodies (150 to 160 kDa) as well as the Fab fragments (~50 kDa) and single-chain variable fragments (~25 kDa). Despite their small size, they possess binding selectivity and affinity toward their target similar to those of whole antibodies. In addition to possessing the advantages of conventional antibodies, nanobodies have additional advantages because of their small size, such as the ability to access enzyme-active sites and receptor clefts, their extreme stability, and the fact that they are easy to manufacture and can be administered by routes other than injection.

Nanobody technology was developed by Ablynx, a biopharmaceutical company based in Ghent, Belgium. Their Nanobody technology was developed based on the discovery of fully functional antibodies in camelidae (camels and llamas) lacking light chains. These heavy-chain antibodies possess a single variable domain and two constant domains, C_H2 and C_H3). Numerous nanobodies are currently being developed for the treatment of gastrointestinal, respiratory, cardiovascular, and dermal diseases. Ablynx's ALX-0081, a therapeutic nanobody for the treatment of cardiovascular diseases, has completed phase I studies and is undergoing phase II clinical trials. ALX-0081 targets von Willebrand factor (vWF), a blood glycoprotein involved in hemostasis, and reduces the risk of thrombosis in patients with acute coronary syndrome and thrombotic thrombocytopenic purpura. ALX-0681 is also an anti-vWF nanobody but is intended for subcutaneous administration. It is currently undergoing phase I studies for assessment of its safety, tolerability, pharmacokinetics, and pharmacodynamics after single and multiple administration [40,41].

3. *Diagnostics*. Tumor-specific monoclonal antibodies can be used to identify and/or distinguish between types of cancers. For example, a tumor-specific antibody introduced by MabCure, Inc. is used to identify ovarian cancer in blood and successfully distinguishes ovarian cancer from benign cancer of ovaries and blood obtained from healthy individuals. MabCure has also identified 10 novel monoclonal antibodies specific to prostate cancer cells. These antibodies are currently undergoing clinical studies for the development of a diagnostic tool to detect prostate cancer in the blood or urine of patients.

Monoclonal antibody–based diagnostic tools can be superior to some existing diagnostic tools, as they are highly specific to the antigens expressed on cancer

cells. For example, prostate-specific antigen (PSA) serum marker is frequently used for the diagnosis of prostate cancer; however, recent studies indicate that this marker may not diagnose prostate cancer accurately, as this test relies on elevated PSA levels, which is a marker of inflammation of the prostate and not specific to prostate cancer [42,43].

4. *Intrabodies*. Two types of intrabodies, also called *intracellular antibodies*, have been recognized: *true intrabodies*, which are expressed and work within the cell, and *retained intrabodies*, extracytoplasmic antibody fragments that are retained within the bounds of a cell membrane by retention and recycling signals. Intrabodies have high specificity and affinity for their antigens and also the ability to bind to various targets, owing to the number of antigen-binding variable domains. Because of these advantages, intrabodies have numerous applications in the field of therapeutic development, target discovery and validation, and agricultural biotechnology.

Anti-NS3 scFvsintrabodies have shown the potential to treat hepatitis C virus (HCV) infection, as these inhibit the HCV NS3/4A serine protease necessary for viral replication. These intrabodies showed promising results in in vitro studies, inhibiting NS3 protease activity and suppressing replication of HCV RNA when expressed intracellularly by DNA transfection in Huh 7 hepatoma cells. Also, anti-NS3 scFvsintrabodies inhibited the replication of replicons A156T and R109K, responsible for conferring resistance to small-molecule antiviral candidates.

Intrabodies specific for H-RAS and cyclin E have shown their usefulness in oncology by blocking transformation in cell culture. Retained intrabodies have also demonstrated their efficacy in treating neurological diseases such as Huntington's, Parkinson's, Alzheimer's, and Prion disease by preventing protein polymerization and/or aggregation. Anti-erbB-2 intrabodies have undergone phase I clinical trials for cancer therapy [44,45].

6 RECENT TRENDS

1. *HuCAL* (Human Combinatorial Antibody Library): a technology designed for the in vitro generation of highly specific and fully human antibodies. These antibodies have high specificity and affinity and are being used for the treatment of various diseases. For example, MorphoSys/Roche HuCAL antibody, gantenerumab, is undergoing phase I clinical trials for the treatment of Alzheimer's disease, and BHQ880 Dickkopf (DKK-1) is currently in phase I/II studies for multiple myeloma indication. Apart from its application in therapeutics, antibodies generated using this technology are also used for diagnostic and research purposes. The advantage of HuCAL technology is that it produces more fully human antibodies than does any other method [46].

2. *BiTe* (bispecific T-cell engaging) *antibodies*: a class of bispecific antibodies that are capable of binding to two different targets simultaneously. BiTe antibodies are composed of variable domains of two monoclonal antibodies linked together by means of a linker sequence and aligned on a single polypeptide chain.

One arm of the BiTe binds to T cells via a CD3 receptor and is common to all BiTeantibodies; the other arm targets specific tumor antigens. BiTe technology is a registered trademark of Micromet, Inc., a biopharmaceutical company.

BiTe antibodies work by forming a link between T cells and cancer cells which causes T cells to produce the cytotoxic proteins perforin and granzymes, which leads to further apoptosis of tumor. Characteristics of BiTe antibodies that differentiate them from other bispecific antibodies include (1) exceptionally high potency of redirected lysis (EC_{50}:0.1 to 50 pmol/L), (2) good activity at a low effector/target ratio, and (3) target cell–dependent activation of B cells.

Two Micromet BiTe antibodies are currently undergoing clinical trials: blinatumomab (MT103), for the treatment of non-Hodgkin lymphoma and lymphoblastic leukemia, and MT110, indicated for the treatment of solid tumors such as colon, breast, prostate, and ovarian tumors. The rest—MT111, MT112, solid tumor BiTe, and multiple myeloma BiTe—are currently in a preclinical development phase [47].

7 FUTURE TRENDS

Future trends in antibody-based therapeutics point at the development of novel synthetic entities resembling antibodies. Researchers at the University of Shizuoka (Japan), Stanford University, and the University of California–Irvine have developed plastic antibodies. These synthetic antibodies are made up of nanoparticles that bind to antigens like natural antibodies and perform similar actions [48]. Researchers at Arizona State University synthesized synthetic antibodies termed *synbodies* by linking the amino acid sequences or peptides by means of a linker. The synbodies are more stable than naturally produced antibodies and will make a good tool for diagnostics [49].

Another arena where development is expected is innovation in antibody engineering for higher therapeutic efficacy and cost-effective manufacturing processes. Identification of new targets and pathways of diseases for the development of antibody therapeutics with novel models of action. In the coming years antibody-based therapeutics is expected to emerge as a strong sector within the pharmaceutical industry, driving the market [50].

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