Recombinant deoxyribonucleic acid (DNA) technology has proven to be a powerful tool to elucidate fundamental biologic processes, disease pathways, and therapeutic targets and has had a major clinical impact in every facet of cardiovascular medicine over the past decade. On a clinical level, recombinant DNA technology has become a cornerstone of biologically targeted cardiovascular therapies, including the creation of new agents to treat acute coronary syndromes, sepsis, anemia, granulocytopenia, and heart failure. The first report of successful molecular cloning was reported in the mid-1970s, which was soon followed by the successful expression of human somatostatin, a 14-amino-acid peptide neurotransmitter, from recombinant DNA cloned into a plasmid in *Escherichia coli*. Before long, human insulin came onto the market as the first commercialized recombinant protein. Recombinant DNA technology has thus evolved into biotechnology, which created an entirely new industry devoted to the cloning and production of recombinant proteins for therapy and diagnosis of human disease. The intersection of biotechnology and clinical medicine has accelerated the development of large-scale production of recombinant proteins for human use, and its clinical applications have produced dozens of recombinant protein drugs for a diverse group of diseases (Table 1-1). Recent advances in protein design and engineering has led to the development of second generation therapeutic proteins that have been engineered to have improved pharmacokinetics, specificity, and side effect profiles. One of the most impressive advances in recent years has been the advent of humanized monoclonal antibodies in clinical practice, which can be administered with minimal or no immune response. Recombinant protein therapeutics has permeated almost every discipline of medical science: hematology, oncology, nephrology, gastroenterology, rheumatology, endocrinology, infectious disease, genetic disease, and cardiovascular disease. As we enter the era of molecular medicine, the recombinant protein technology will continue to shape the practice of medicine.

RECOMBINANT PROTEIN PRODUCTION: EXPRESSION SYSTEMS

The production of recombinant proteins is based on the introduction of a cloned complementary DNA (cDNA), which encodes the particular protein of interest into a host cell and essentially provides the molecular machinery for protein synthesis (for a review, see reference 2). A wide variety of expression systems are currently available: bacteria, yeast, insect, mammalian cells, and transgenic animals. Each expression system has inherent advantages and disadvantages, and the choice of the expression system depends on many factors, including intrinsic structural features of the protein of interest, the stability of the protein, and the projected dose of protein per patient (Table 1-2).

Bacterial cells, most commonly *E. coli*, are the most cost-effective among all expression systems and can be used to produce a large amount of proteins in a relatively short period. On the other hand, the bacterial cell machinery lacks the capability to complete critical aspects of posttranslational protein modifications that can be critical for therapeutic efficacy. In addition, foreign proteins expressed in bacteria are often insoluble, thus forming dense aggregates called inclusion bodies, necessitating a cumbersome denaturation/renaturation process to recover active protein.

Yeast cells maintain economic advantages comparable to those of bacteria and are capable of performing most of the eukaryotic posttranslational modifications, including phosphorylation, glycosylation, disulfide bond formation, and proteolytic cleavage of inactive precursors. Because of the sophisticated protein processing of yeast cells, recombinant proteins that are insoluble when expressed in bacteria may be soluble if the yeast system is used. Yeast cells can also be induced to secrete recombinant proteins into the growth medium for harvesting. The quantity of recombinant proteins expressed in yeast cells, however, is often limited by active proteases present in yeast cells that degrade foreign proteins. This problem may be circumvented by construction of yeast strains lacking the protease genes. Other disadvantages
of the yeast system include overglycosylation of glycoproteins, which may alter the activity of the expressed proteins, vector instability, and entrapment of secreted proteins in the periplasmic space.

The baculovirus system uses viral vectors that infect and multiply in cultured insect cells to express foreign proteins. Baculovirus expression of foreign genes in insect cells permits protein folding, posttranslational modification, and oligomerization seen in mammalian cells. Recombinant proteins can be produced in large quantities either within the cells or secreted into the culture medium. One of the major disadvantages of the baculovirus system is that foreign proteins are expressed during acute lytic infection of insect cells, resulting in short production period and cell death. In addition, generation and transfection of baculovirus vectors in insect cells may be difficult, and some proteins may not undergo proper modifications. Insect cells also grow more slowly and are more expensive than bacterial and yeast cells.

Mammalian cells, although costly, have recently become one of the most popular and valuable expression systems. Mammalian cells, such as Chinese

<table>
<thead>
<tr>
<th>TABLE 1-1 RECOMBINANT PROTEIN DRUGS</th>
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<tbody>
<tr>
<td><strong>Product</strong></td>
</tr>
<tr>
<td>Humulin</td>
</tr>
<tr>
<td>Promotrin (somatrem)</td>
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<tr>
<td>Roleron-A</td>
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<tr>
<td>Intron A</td>
</tr>
<tr>
<td>Recombivax HB</td>
</tr>
<tr>
<td>Activase (alteplase)</td>
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<tr>
<td>Epogen</td>
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<tr>
<td>Alleron N</td>
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<tr>
<td>Actimmune</td>
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<tr>
<td>Neupogen</td>
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<tr>
<td>Leukine</td>
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<tr>
<td>Proleukin</td>
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<tr>
<td>Recombinate</td>
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<tr>
<td>KoGENate</td>
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<tr>
<td>Betasert</td>
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<tr>
<td>Pulmozyme (dornase alfa)</td>
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<tr>
<td>Nutropin (somatropin)</td>
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<tr>
<td>Cerezyme (crecades)</td>
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<tr>
<td>Nutropin AQ</td>
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<tr>
<td>Humalog</td>
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<tr>
<td>Avonex</td>
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<tr>
<td>Retavase (reteplase)</td>
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<tr>
<td>BenefIX</td>
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<tr>
<td>Intergen</td>
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<tr>
<td>Neumega</td>
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<tr>
<td>Regranex Gel</td>
</tr>
<tr>
<td>Enbrel (etanercept)</td>
</tr>
<tr>
<td>GlucAg</td>
</tr>
<tr>
<td>NovoSeven</td>
</tr>
<tr>
<td>Nutropin Depot</td>
</tr>
<tr>
<td>Refacto</td>
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<tr>
<td>TNKase (tenecteplase)</td>
</tr>
<tr>
<td>Aranesp (darbepoetin alfa)</td>
</tr>
<tr>
<td>Kineret (anakinra)</td>
</tr>
<tr>
<td>Natrecor (nestrinide)</td>
</tr>
<tr>
<td>Xigris (drotrecogin alfa)</td>
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<tr>
<td>Peg-Intron</td>
</tr>
</tbody>
</table>

**Recombinant Monoclonal Antibody (mAb)**

<table>
<thead>
<tr>
<th>Product</th>
<th>Category</th>
<th>First FDA approval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orthoclone OKT3</td>
<td>Anti-CD3 mouse mAb</td>
<td>Reversal of acute kidney transplant rejection</td>
</tr>
<tr>
<td>OrthoPro (abciximab)</td>
<td>Anti-GPIIb/IIIa chimera</td>
<td>Refractory UA when PCI is delayed</td>
</tr>
<tr>
<td>Rituxan (rituximab)</td>
<td>Anti-CD20 chimera</td>
<td>Refractory RA; other</td>
</tr>
<tr>
<td>Zenapax (daclizumab)</td>
<td>Anti-Tac humanized mAb</td>
<td>Prevention of acute kidney transplant rejection</td>
</tr>
<tr>
<td>Herceptin (trastuzumab)</td>
<td>Anti-HER2 humanized mAb</td>
<td>Metastatic breast cancer</td>
</tr>
<tr>
<td>Remicade (infliximab)</td>
<td>Anti-TNFα chimera</td>
<td>Crohn's disease</td>
</tr>
<tr>
<td>Synagis (palivizumab)</td>
<td>Anti-RSV humanized mAb</td>
<td>Prophylaxis of RSV infection in pediatrics</td>
</tr>
<tr>
<td>Mylotarg (gemtuzumab)</td>
<td>Anti-CD33 humanized mAb with calicheamicin</td>
<td>Acute myeloid leukemia</td>
</tr>
<tr>
<td>Campath (alemtuzumab)</td>
<td>Anti-CD52 humanized mAb</td>
<td>B-cell chronic lymphocytic leukemia</td>
</tr>
</tbody>
</table>

G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; II, interleukin; PCI, percutaneous transluminal coronary angioplasty; RSV, respiratory syncytial virus; SC, subcutaneous; TNF, tumor necrosis factor; t-PA, tissue plasminogen activator; UA, unstable angina.
Hamster Ovary (CHO) cells, produce necessary post-translational modifications and recognize the same synthesis and processing signals found in the original organism. The major disadvantages of the mammalian system are low expression levels, long generation times, and high costs. Mammalian cell transfections are also less efficient than in bacteria or yeast, which further promote lower overall expression levels in the mammalian system.

Alternatively, the production of recombinant proteins in transgenic animals can achieve cost-effectiveness while maintaining favorable properties of the mammalian cell expression system. For example, transgenic goats express 1 to 3 g/L of tissue plasminogen activator (t-PA) in milk. A major drawback of the transgenic animal expression system is that it takes months and sometimes years to raise the sufficient number of transgenic animals from the founder to produce the amount of proteins adequate for commercialization.

### RECOMBINANT PROTEIN DRUGS

#### Nonmonoclonal Antibody Drugs

Most recombinant protein drugs currently on the market are naturally occurring human proteins, including coagulation factors, growth hormone, erythropoietin, colony-stimulating factors, t-PA, and insulin. Furthermore, clinically useful proteins have been engineered to improve the original function toward specific therapeutic endpoints, and these engineered proteins account for a small portion of the recombinant protein drugs on the market. The functional improvements are accomplished through deletion/substitution of functional domains, site-specific point mutations, alteration of glycosylation sites, and/or fusion with other functional proteins. In addition to the traditional recombinant DNA technology, the phage display technology has allowed determination of effective domain sequences that possess higher binding affinity than the native proteins while retaining their normal biological function and pharmacokinetics (see the section on fully human monoclonal antibodies). This group of engineered proteins includes hepatitis vaccines, interferon variants, t-PA variants, and fusion proteins.

#### Insulin

Insulin is a classic example of a naturally occurring protein produced by recombinant technology. Human insulin was the first recombinant protein commercially produced for therapeutic use. Before the availability of recombinant insulin, diabetic patients relied on insulin purified from the pancreases of pigs and cows, which occasionally resulted in serious immune reactions. Recombinant human insulin has dramatically improved the therapy for diabetes. Human insulin is expressed as a proinsulin that subsequently underwent enzymatic cleavage to form the active insulin molecule, but currently it is produced by expressing A and B chains separately, then refolding them into a mature insulin molecule (Figure 1-1).

#### Somatropin

Somatropin is the recombinant human growth hormone (rhGH), identical to the pituitary-derived human growth hormone with respect to amino acid sequence (191 amino acids, 22 kDa). Somatropin is synthesized in E. coli as a precursor consisting of the GH molecule conjugated with a secretion signal from an E. coli protein, which directs the precursor to the plasma membrane of E. coli. The signal sequence is then removed, and the GH protein is secreted into the periplasm so that the protein is folded appropriately as it is expressed. Somatropin is indicated for the treatment of growth failure resulting

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**TABLE 1-2 COMPARISON OF EXPRESSION SYSTEMS**

<table>
<thead>
<tr>
<th>Expression System</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteria</td>
<td>Short generation times, Large quantities of product, Low cost</td>
<td>Lack of posttranslational modifications, Formation of inclusion bodies, Unsuitable for large or complex proteins</td>
</tr>
<tr>
<td>Yeast</td>
<td>Some eukaryotic posttranslational modifications, Moderately short generation times</td>
<td>Product quantity limited by protease degradation, Overglycosylation, Vector instability, Product entrapment in periplasmic space</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>Large quantities of product, Some eukaryotic posttranslational modifications, Proper protein folding</td>
<td>Difficult to handle, More expensive than bacteria or yeast, Long generation times, Unsuitable for proteins with repetitive sequences</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>Eukaryotic posttranslational modifications</td>
<td>Long generation times, Difficult to scale up, High cost, Time-consuming</td>
</tr>
<tr>
<td>Transgenic animals</td>
<td>Enormous quantities of product, Eukaryotic posttranslational modifications</td>
<td>Can be cost-effective</td>
</tr>
</tbody>
</table>
provided a way to cleave off the synthesized so that it started with a methionine codon. This setup inside the bacterial cells. The cells were harvested, and each recombinant insulin. A and B chains were purified and then mixed together to form active cleaved further because they did not contain internal methionines. The CNBr cleaved it into many small peptides. The insulin chains were not because CNBr treatment resulted in cleavage of peptide bonds after all methionines. In this way, the natural insulin peptides were obtained. Because β-gal contains other methionine residues, CNBr treatment cleaved it into many small peptides. The insulin chains were not cleaved further because they did not contain internal methionines. The A and B chains were purified and then mixed together to form active recombinant insulin. (From Recombinant DNA, by J.D. Watson, M. Gilman, J. Wilkonski, M. Zoller ©1992, 1983 by J.D. Watson, M. Gilman, J. Wilkonski, M. Zoller. Used with the permission of W.H. Freeman and Company, Cold Spring Harbor, NY)

Recombinant human growth hormone has been investigated in clinical studies for patients with chronic heart failure. Serum levels of insulin-like growth factor (IGF-I), reflecting endogenous GH secretion, are diminished in relation to the severity of heart failure in patients with dilated cardiomyopathy, and administration of rhGH increases the IGF-I levels, resulting in significant improvement of ejection fraction. In patients with idiopathic dilated cardiomyopathy, rhGH increases myocardial mass and VO₂ max, while it reduces the left ventricular chamber size; myocardial sympathetic drive; serum levels of aldosterone; and proinflammatory cytokines including tumor necrosis factor (TNF-α), its soluble receptors (sTNF-RI, sTNF-RII), interleukin-6 (IL-6), soluble IL-6 receptor (sIL-6R), and soluble Fas/FasL system. However, the results from randomized studies have been conflicting. In some studies, rhGH improved hemodynamics, myocardial energy metabolism, and clinical performance whereas other studies showed no benefits in improving clinical status, cardiac function, or neuroendocrine activation in patients with dilated cardiomyopathy, despite a significant increase in left ventricular mass. These contradictory results may be the result of variable levels of IGF-I response to rhGH administration.

Recombinant Human Insulin-like Growth Factor-I
Recombinant human insulin-like growth factor (rhIGF-I), which contains 70 amino acid residues (7.5 kDa), is produced in a variety of expression systems. Endogenous IGF-I may play a pivotal role in compensated heart failure, because the serum levels of IGF-I are elevated in mild to moderate heart failure (NYHA class I and II) but not in severe heart failure (NYHA class III and IV). Although it has not been approved for clinical use, rhIGF-I has vasodilatory and positive inotropic effects and has been tested in human patients with heart failure. In healthy individuals, rhIGF-I significantly increase stroke volume, cardiac output, and ejection fraction without increasing heart rate at rest or during exercise. In patients with chronic heart failure, rhIGF-I acutely increase stroke volume and cardiac index and decreases pulmonary artery wedge pressure and systemic vascular resistance. A number of clinical studies of rhIGF-I and rhGH indicate significant potential of rhIGF-I as a drug to treat chronic heart failure. Further studies to establish long-term efficacy and safety of rhIGF-I are warranted.

Nesiritide
Nesiritide is the recombinant human B-type natriuretic peptide (rhBNP) produced in E. coli. Nesiritide (32 amino acids, 3.5 kDa) is identical in amino acid sequence to the naturally occurring hBNP produced by ventricular cardiomyocytes. Not only does rhBNP have diuretic and natriuretic actions, but it also binds to the particulate guanylate cyclase receptors of vascular smooth muscle and endothelial cells, causing cyclic guanosine monophosphate (GMP)-mediated smooth muscle relaxation and vasodilation. Nesiritide is indicated for intravenous use in patients with acutely decompensated congestive heart failure with dyspnea at rest or minimal activity. In clini-
clinical trials, nesiritide reduced pulmonary capillary wedge pressure and improved dyspnea in this population.\textsuperscript{18}

**Thrombolytic Agents**

Thrombolytic agents provide a classic example of the power of engineered recombinant proteins for human cardiovascular disease. Currently, there are three distinct generations of thrombolytic agents. The first-generation thrombolytics—streptokinase, anistreplase, and urokinase—are not fibrin-specific and activate plasminogen systemically, which may lead to a systemic lytic state. The second-generation thrombolytics include alteplase (rt-PA), saruplase (scu-PA), and duteplase; and the third-generation includes reteplase (r-PA), lanetoplase (n-PA), and tenecteplase (TNK-rt-PA). The second- and third-generation thrombolytics have also been designated as fibrinolytics, because they preferentially activate plasminogen at the fibrin clot, although systemic activation of plasminogen does occur with clinical doses.

**Alteplase (rt-PA)**

Alteplase (rt-PA) is a recombinant protein of the naturally occurring t-PA. After human t-PA was cloned and expressed in *E. coli* and in mammalian cells,\textsuperscript{19} it became the first commercially available recombinant protein drug that was entirely produced in mammalian cells (Figure 1-2). Since then, alteplase has been widely used as a fibrinolytic agent for patients with acute myocardial infarction.\textsuperscript{20}

Human t-PA is a 527-amino-acid, single-chain serine protease (70 kDa) (Figure 1-3). It converts plasminogen into the active serine protease plasmin, which degrades fibrin meshwork in clots. By itself, t-PA is a weak enzyme, but fibrin remarkably enhances its enzymatic potency.\textsuperscript{21}

The t-PA molecule contains multiple distinct domains, and the functions of these domains have been identified by detailed structural-functional analysis with deletion mutagenesis.\textsuperscript{22} The finger domain is the high-affinity binding site to fibrin, whereas the epidermal growth factor (EGF) and kringle 1 domains affect receptor binding in the liver and rapid serum clearance. The EGF domain interacts with calcium-dependent receptors on the liver parenchymal cells, and the kringle 1 domain contains the high mannose-type carbohydrate side-chain at Asn\textsuperscript{117} that binds to mannose receptors on the liver endothelial cells. The glycosylation at Asn\textsuperscript{117} is responsible for rapid clearance of t-PA by the liver. The kringle 2 domain binds to lysin and facilitates conversion of plasminogen to plasmin. The lysin-binding site of the kringle 2 domain, along with the finger domain, also mediates fibrin binding. The protease domain is a plasminogen-specific serine protease and contains the binding site for plasminogen activator inhibitor (PAI)-1.

As a therapeutic agent for acute myocardial infarction, t-PA has several unfavorable properties. Its short half-life (2 to 5 minutes) necessitates continuous intravenous infusion at relatively high doses for as long as 90 minutes, and its slow onset of action may allow for expansion of myocardial damage. The use of t-PA also requires adjunct heparin infusion to prevent reocclusion, which is caused by thrombin released during fibrinolysis. Moreover, its fib-
rin-specificity is not clinically apparent. In a large-scale clinical trial, the rate of hemorrhagic stroke was significantly higher with accelerated t-PA versus streptokinase (0.72% vs. 0.54%, P = 0.03). These shortcomings have prompted investigators to engineer the original t-PA molecule to create mutants with higher fibrin-specificity, more protease potency, slower clearance from the circulation, and more resistance to plasma inhibitors.

Repteplase (r-PA)

Reteplase (r-PA, 39 kDa) is a single-chain non-glycosylated deletion mutant of human t-PA containing the kringle 2 and protease domains but lacking the kringle 1, finger, and EGF domains (Figure 1-4). Reteplase is designed to contain the minimum number of necessary functional domains and therefore has an improved solubility and is easily expressed in E. coli. Reteplase is isolated as inactive inclusion bodies from E. coli, converted into its active form by an in vitro folding process, and purified by chromatographic separation.

Compared with t-PA, reteplase has more fibrin-specificity, a longer half-life (14 to 18 minutes), enhanced fibrinolytic potency, and a lower affinity for endothelial cells. The low affinity for endothelial cells and monocytes is most likely because of the absence of the finger and EGF domains, which are apparently involved in the interaction with endothelial cell receptors. Resistance to PAI-1 is not affected by these deletions. Development of anti-reteplase antibodies in patients treated with reteplase has not been reported.

In contrast to alteplase, reteplase is administered as a double-bolus injection at 30 minutes apart because its longer half-life. Each bolus is administered as an intravenous injection over 2 minutes. Based on a number of clinical trials, reteplase is considered therapeutically similar to alteplase, and the double-bolus administration is an advantage over alteplase.

Lanoteplase (n-PA)

Lanoteplase is a deletion mutant of t-PA that lacks the finger, EGF domains, and Asn glycosylation. These modifications result in enhanced fibrin binding, more fibrinolytic potency, and a longer half-life (23 minutes). Serum PAI activity is significantly lower with lanoteplase.
than t-PA (P < 0.01), which may contribute to its long half-life and potency. Anti-tenecteplase antibodies have not been detected in various animal models. Despite its promising preliminary results, the development of lanetoplase was discontinued because of a higher incidence of intracranial hemorrhage than t-PA in a clinical trial (1.12% vs. 0.64%, P = 0.004).

Tenecteplase (TNK-rt-PA)

Tenecteplase is a triple mutant of t-PA expressed in the CHO cells. The Asn117 glycosylation site is deleted by substituting Asn117 with Gln (N117Q), and another glycosylation site is created in the same domain by replacing Thr103 with Asn (T103N). This change in the glycosylation locus significantly increases the fibrin binding and serum half-life (10 to 24 minutes). In addition, the sequence Lys296-His297-Arg298-Arg299 is replaced by four successive alanines, which increases resistance to PAI-1 and enhances fibrin specificity. This is accomplished through alanine-scanning mutagenesis, in which the amino acids located at randomly chosen points in the t-PA sequence are replaced with alanine with repeated analysis of the newly created mutant molecules. Tenecteplase has more fibrinolytic potency than t-PA, and no immunogenic response has been reported. These favorable properties give tenecteplase an advantage of a single bolus injection with the efficacy and safety comparable to that of accelerated t-PA. A large-scale clinical trial has shown that for the tenecteplase and t-PA groups, the rates of 30-day mortality (6.18% vs. 6.15%), and intracranial hemorrhage (0.93% vs. 0.94%) were almost identical. The tenecteplase group had fewer non-cerebral bleeding complications (26.43% vs. 28.95%, P = 0.0003) and less frequent need for blood transfusion (4.25% vs. 5.49%, P = 0.0002) compared with the t-PA group. The combination of tenecteplase plus enoxaparin or abciximab reduces the frequency of ischemic complications of acute myocardial infarction compared with that of tenecteplase plus unfractionated heparin.

Etanercept

Etanercept is a dimeric fusion protein composed of the extracellular ligand-binding portion of the human TNF p75 receptor and the Fc portion of human IgG1. Each etanercept molecule (934 amino acids, 150 kDa) contains two TNF p75 receptors and one Fc portion. The Fc component of etanercept contains the hinge region, the C\textsubscript{\text{H}2} and C\textsubscript{\text{H}3} domains of IgG\textsubscript{1} (see the section on molecular structure of antibodies). Etanercept is produced in the CHO mammalian cell expression system.

Etanercept has been investigated for treatment of heart failure. The efficacy of etanercept to inhibit the negative inotropic effects of TNF-α shown in preclinical studies was confirmed in small, short-term clinical studies. Based on these promising results, two large-scale randomized clinical trials, the RENAISSANCE trial in North America and the RECOVER trial in Europe and Australia, were initiated to evaluate the efficacy of etanercept in patients with moderate to severe heart failure. The primary end point of the RENAISSANCE and RECOVER was a clinical composite. The RENEWAL trial, which used the pooled data from the other two trials, evaluated the all-cause mortality and hospitalization for heart failure as the primary end point. These trials, however, were terminated prematurely because preliminary analysis of the data showed no benefit for etanercept on
the clinical composite end point in RENAISSANCE and RECOVER and no benefit for all-cause mortality and heart failure hospitalization in RENEWAL.45

Monoclonal Antibody Drugs

Since the 19th century, antibodies have been widely used for treatment and prophylaxis of disease in the form of animal-derived antisera. However, the efficacy of antiserum therapy was severely limited, because antiserum contains polyclonal antibodies, and only some of these antibodies bind to the target antigens. In addition, antiserum therapy was complicated by the symptoms of serum sickness, ranging from minor joint pain and fever to lethal anaphylactic shock. Serum sickness was caused by immune reactions against a number of animal-derived antibodies and nonantibody proteins present in the serum.

Over the last two decades, a series of technical breakthroughs have accelerated the evolution of antibody-based therapeutics. Recombinant DNA technology and hybridoma technology, both born in the mid-1970s, gave rise to antibody engineering, a highly specialized area of protein engineering devoted to the development of monoclonal antibodies (mAbs). Antibody engineering has resulted in the creation of fusion, chimeric, and humanized antibodies that are currently applied to the treatment of a wide spectrum of human diseases. Finally, phage display technology has replaced hybridoma technology, allowing construction of entirely human monoclonal antibodies with the desired antigenic specificity.

Molecular Structure of Antibodies

Antibodies, also known as immunoglobulins (Igs), are large glycoprotein molecules produced by B lymphocytes. The most common human Ig, IgG, is shaped like the capital letter "Y" in which an antigen-binding site is at the end of the two arms, and an effector site is on the leg (Figure 1-5). The IgG is therefore bivalent. An IgG molecule consists of four protein chains, a pair of heavy chains and a pair of light chains, linked by disulfide bonds. Each chain consists of domains of approximately 110 amino acids in length; the light chain contains two domains, whereas the heavy chain has four. Antibodies can also be digested with proteases to release different fragments called Fv (variable fragment), Fab (antigen-binding fragment), and Fc (crystallization fragment). In addition, there are different isotypes of each chain: \( \kappa \) and \( \lambda \) in the light chain, and \( \gamma, \alpha, \delta, \mu, \) and \( \varepsilon \) in the heavy chain. Depending on the isotype of the heavy chain, human Igs are divided into five different classes, IgG1–4, IgA1–2, IgM, IgD, and IgE. Each class of heavy chain can combine with either of the light chains. These different antibody classes are variations of IgG with a different number of chains or a different number of constant regions and sometimes with an additional J chain.

An antigen-binding site consists of the variable domains of both the light and heavy chains, created by somatic recombination and mutagenesis. The variable domains are highly diverse in amino acid sequence and thus confer specificity against a variety of antigenic epitopes. In contrast to the variable domains (\( V_L, V_H \)), the other domains contain a stable amino acid sequence, and are called constant domains (\( C_L, C_{H1}, C_{H2}, C_{H3} \)). Only a part of the amino acids in the variable region actually contacts the antigens, and these regions are called complementarity-determining regions (CDRs). The remaining components of the variable region serve as a scaffold to hold the CDRs in the right positions. There are three CDRs on the variable domain of each chain; therefore a total of six CDRs exist in an antigen-binding site (\( CDR_{H1-3}, CDR_{L1-3} \)). The length and composition of the amino acid sequence at the CDRs are hypervariable among different antibodies and are responsible for the specificity and affinity of the antibodies to their target antigens.

The effector functions are mediated by the constant (Fc) regions of the antibody. The Fc portion binds to the Fc receptors on the surface of the effector cells that induce antibody-dependent cellular cytotoxicity (ADCC). In addition, a different region of the Fc portion of the antibody molecule binds to C1q and initiates the classical complement pathway, which leads to complement-mediated cytolysis.

Mouse Monoclonal Antibodies

The advent of the hybridoma technology (Figure 1-6) has revolutionized the scientific study of Igs, fostered their clinical application, and has set the ground for the development of antibody engineering.46 The hybridoma technology has allowed large-scale production of mAbs that specifically bind to almost any target antigen of interest.

Although the hybridoma technology has provided mAbs that are currently playing a major role in biomedical research and clinical medicine, the application of hybridoma-derived mAbs for therapeutic use is limited by multiple difficulties. Because most hybridomas are of mouse origin, hybridoma-derived mAbs are not identical to human antibodies. When mouse mAbs are injected into humans, the patients develop anti-mouse mAb antibodies that accelerate their clearance. This human anti-mouse antibody (HAMA) response can occur within

*FIGURE 1-5. Structure of IgG. Gray ovals represent variable domains (\( V_L, V_H \)), and white ovals represent constant domains (\( C_L, C_{H1}, C_{H2}, \) and \( C_{H3} \)).*
functions of the immune system, because rodent Fc fragments do not interact well with human Fc-dependent effector mechanisms.

**Muromonab-CD3**

Muromonab-CD3 (OKT3) was the first therapeutic mAb in the history of medicine. Muromonab-CD3 is a mouse mAb against the CD3 molecules that form a portion of the human T cell antigen-receptor complex. Muromonab-CD3 binds to all mature T cells, functioning as an immunosuppressant. Although muromonab-CD3 is widely used in reversing rejection of transplanted organs, including heart, kidneys, and liver, the period of use is limited by the development of the HAMA response.

**Recombinant Antibody Fragments**

Detailed sequence-structural analysis of antibody molecules has been made available through hybridoma and recombinant DNA technology, which has resulted in creation of antibody fragments of the smallest possible size that maintain the original antigen-binding capacity. Various recombinant antibody fragments with antigen-binding sites have been expressed in prokaryotes, including Fv, single-chain Fv (scFv), and Fab (Figure 1-7). The Fv module (25 kDa) is the smallest monovalent antigen-binding unit of an antibody and consists of V<sub>H</sub> and V<sub>L</sub> domains linked by disulfide bonds. The scFv module is an Fv module with an additional peptide that covalently links the V<sub>H</sub> and V<sub>L</sub> domains. The peptide linker is usually 15 to 20 amino acids long and is introduced at the cDNA level. These fragments are attached to a bacterial leader sequence that transports the protein to the periplasmic space, where the V<sub>H</sub> and V<sub>L</sub> domains fold into active protein with disulfide bond formation between the two domains.

Because of their small size (30 kDa), scFv fragments are easily expressed in bacterial cells and are subject to various engineering efforts using classic recombinant DNA techniques. Other fragments can be genetically combined with scFv fragments to produce _multivalent antibodies_ (e.g., bivalent, trivalent, tetravalent antibodies) with increased affinity and tissue targeting or _multispecific antibodies_ (e.g., bispecific, trispecific, tetraspecific antibodies) that recognize different antigens, thus allowing an antibody to bridge different antigens (see Figure 1-7). An example of a bispecific antibody is an anti-T cell receptor antibody fragment attached to a fragment with specificity for a viral, parasitic, or tumor antigen, which can bring the cytotoxic T cell directly to the antigens. Moreover, the antibody fragments can be fused genetically with toxins, enzymes, or cytokines to form _fusion antibodies_ that may exhibit more potent effector functions than the Fc-dependent effector mechanisms, which are lacking in the small antibody fragments. Fusion antibodies that carry human TNF with specificity for colorectal carcinoma cell antigens are being developed. Despite the potential significance of fusion antibodies for therapeutic use, the pharmacologic and pharmacokinetic properties of fusion antibodies _in vivo_ are affected by various parameters and are often unpredictable. Another therapeutic application is the intracellular expression of antigen recognition.
domain of immunoglobulins to inhibit intracellular processes. The cDNA of these *intrabodies* are cloned into gene transfer vectors and are introduced in the target cells. The intrabodies can be directed to different intracellular compartments by adding various signal sequences and can be used to inhibit intracellular enzymes, transcription factors, and receptors.

**Chimeric Monoclonal Antibodies**

To overcome the shortcomings of conventional mouse mAb therapy, hybrids of mouse and human antibodies have been produced (*chimeric monoclonal antibodies*) (Figure 1-8). Chimeric mAbs are constructed by ligating the cDNA fragment that encodes the xenogenic (e.g., mouse) V<sub>L</sub> and V<sub>H</sub> domains to the fragment that encodes the Fc domain from a human antibody. In general, chimeric mAbs contain approximately 33% mouse protein and 67% human protein. Therefore, the final hybrid antibody products have the mouse variable regions that have the desired antigen specificity and the human constant regions that activate effector mechanisms. Construction of chimeric mAbs is relatively easy compared with constructing humanized mAbs. Chimeric mAbs exhibit reduced immunogenicity compared with mouse mAbs while retaining high binding affinity and specificity. However, some patients still experience variable degrees of human anti-chimeric antibody (HACA) immune response, which may reduce the efficacy of the mAb as a drug.

**Abciximab**

Abciximab is the first human-murine chimeric mAb commercially produced for therapeutic use. Abciximab is the Fab fragment (48 kDa) of the mAbs that binds selectively to the glycoprotein (GP) IIb/IIIa receptors of human platelets. The GP IIb/IIIa (α<sub>IIb</sub>β<sub>3</sub>) is a member of the integrin family of adhesion receptors and is the major platelet surface receptor involved in platelet aggregation. Abciximab inhibits platelet aggregation by preventing the binding of fibrinogen, von Willebrand factor, and other adhesive molecules to GPIIb/IIIa receptors on activated platelets. Abciximab also binds to the Mac-1 (CD11b/CD18) on leukocytes and the vitronectin (α<sub>v</sub>β<sub>3</sub>) on platelets, vascular endothelial cells, and smooth muscle cells, although the relationship of binding characteristics to Mac-1 and vitronectin to clinical efficacy remains uncertain. Abciximab is devoid of the Fc fragment to decrease immunogenicity and, therefore, cannot activate effector mechanisms. Abciximab contains the murine V<sub>L</sub> and V<sub>H</sub> regions that confer antibody specificity, and the human C<sub>L</sub> and C<sub>H1</sub> regions derived from human IgG<sub>1</sub>. Abciximab is produced by continuous perfusion in mammalian cell culture. The Fc fragment is cleaved with papain, and the Fab fragment is purified from the cell culture supernatant by column chromatography.

Abciximab is indicated as an adjunct to coronary angioplasty for the prevention of acute cardiac ischemic complications in patients at high risk for the sudden closure of the revascularized target vessel.

**Infliximab**

Infliximab is a chimeric IgG<sub>1</sub> mAb with a molecular weight of 149 kDa. It is composed of human constant regions and murine variable regions that specifically bind to human TNF-α. Infliximab is produced by a recombinant cell line cultured by continuous perfusion and is purified by a series of steps that includes measures to inactivate and remove viruses.
The efficacy of infliximab was first tested in Crohn’s disease. A randomized clinical study has shown that infliximab is effective in treatment of draining fistulas resulting from Crohn’s disease. Infliximab was also evaluated in active rheumatoid arthritis, and the combination of infliximab and methotrexate significantly and persistently reduced the signs and symptoms, improved the quality of life, and delayed the progression of joint damage compared with methotrexate therapy alone. Infliximab was subsequently examined in a number of inflammatory diseases, including psoriatic arthritis, psoriasis, refractory sarcoidosis, ulcerative colitis, Sjögren’s syndrome, and ankylosing spondylitis, and yielded favorable results. Nevertheless, results from clinical studies evaluating infliximab as a therapy for moderate to advanced heart failure have been disappointing. A randomized phase II clinical study (ATTACH trial) demonstrated a significant dose-related increase in death and heart failure hospitalizations with infliximab compared with a placebo. The administration of infliximab is associated with serious infections, including bacterial pneumonia, sepsis, histoplasmosis, listeriosis, coccidiomycosis, pneumocystosis, and tuberculosis. In addition, treatment with infliximab can result in the formation of antibodies against infliximab. The development of anti-infliximab antibodies is associated with an increased risk of infusion reactions and a reduced duration of response to treatment. Concomitant immunosuppressive therapy may reduce the magnitude of the immunogenic response.

Humanized Monoclonal Antibodies

Humanized mAbs have been constructed to minimize the HAMA and HACA responses triggered with mouse and chimeric mAbs while maintaining high binding specificity to the desired antigen. In humanized mAbs, only the CDRs derive from mice or other xenogenic sources (5% to 10%), and the rest of the antibody domains (90% to 95%) are of human origin. Humanization requires grafting the CDR amino acid sequences from mouse mAbs into human antibodies using in vitro mutagenesis; therefore, humanized mAbs are also called CDR-grafted or reshaped mAbs. Although the recombinant DNA techniques to create humanized mAbs are relatively straightforward, mere grafting of the mouse CDRs into human antibodies does not always produce the antigen specificity and affinity of the original mouse mAb. The design of the engineered mAb has been found to be critical in reconstituting the properties of the original molecule. The designing process requires meticulous analysis of the sequence, structure, and glycosylations of the antigen-binding site of the original mouse mAb. An appropriate human framework that anchors mouse CDRs is selected out of numerous candidates, and the extent of mouse CDRs to be grafted into the human framework is determined following repeated construction and testing of different prototype antibodies. Therefore, the humanization process is technically challenging and often arduous and is still an area of active research efforts. To date, humanized mAbs were found to trigger minimal or no immune response in humans. However, all CDRs are unique and may contain components that are potentially antigenic regardless of production method. In fact, a small number of patients have developed human anti-human antibodies (HAHA) against a conformational antigenic determinant of the humanized monoclonal antibodies.
Daclizumab

Daclizumab is an immunosuppressive, humanized IgG mAb (144 kDa) that binds specifically to the α subunit (p55α, CD25, or Tac subunit) of the human high-affinity IL-2 receptor that is expressed on the surface of activated lymphocytes. The human sequences derive from the constant regions of human IgG1 and the variable regions of the Eu myeloma antibody. The murine sequences derive from the CDRs of a murine anti-Tac antibody. Daclizumab is currently indicated for prophylaxis of acute organ

FIGURE 1-9. Phage display technology. In the top panel, a repertoire of single-chain Fv genes (scFv) is generated with a polymerase chain reaction. The repertoires are constructed either from V₅₇ and V genes that have been rearranged in vivo or from V gene segments that are rearranged in vitro (not shown). The repertoire of scFv genes is cloned into a phage vector in a way that fuses the scFv gene to a gene (gene III) that encodes a protein (pIII) expressed on the phage surface. In the middle panel, the vector directs E. coli to produce phage antibodies, which have on their surface a functional scFv fused to pIII. Inside each phage antibody is the vector DNA containing the gene for the scFv. Phage antibodies binding a specific antigen can be separated from nonbinding phage antibodies by affinity chromatography on immobilized antigen (bottom panel). A single round of selection increases the number of antigen-binding phage antibodies by a factor ranging from 20 to 1000, depending on the affinity of the antibody. Eluted phage antibodies are used to infect E. coli, which then produce more phage antibodies for the next round of selection. Repeated rounds of selection make it possible to isolate antigen-binding phage antibodies that were originally present at frequencies of less than 1 in a billion. (From Marks C, Marks JD: Phage libraries: A new route to clinically useful antibodies. N Engl J Med 1996;335:730. Copyright © 1996 Massachusetts Medical Society. All rights Reserved.)
rejection in patients receiving renal transplants as part of an immunosuppressive regimen that includes cyclosporine and corticosteroids. In a randomized clinical trial, daclizumab significantly reduced the rate of biopsy-proven rejection in patients who received renal transplant compared with the placebo group\(^5\) (28\% vs. 47\%, \(P = 0.001\)). Daclizumab was also evaluated in cardiac transplantation, and it significantly reduced the rate of acute rejection compared with a placebo\(^5\) (18\% vs. 63\%, \(P = 0.04\)).

**Trastuzumab**

Trastuzumab is a humanized mAb that selectively binds to the extracellular domain of the human epidermal growth factor receptor 2 protein, HER2 (ErbB2). The antibody is an IgG\(_1\) that contains human framework regions with CDRs of a murine antibody that binds to HER2. Trastuzumab is produced by a CHO cell suspension culture in a nutrient medium. Trastuzumab is indicated for the treatment of HER2 overexpressing metastatic breast cancer, which comprises 25\% to 30\% of breast cancers.

In patients with metastatic breast cancer, trastuzumab delayed the time to disease progression (median, 7.4 vs. 4.6 months, \(P < 0.001\)), increased the rate of objective response (50\% vs. 32\%, \(P < 0.001\)), prolonged the duration of response (median, 9.1 vs. 6.1 months, \(P < 0.001\)), decreased 1-year mortality (22\% vs. 33\%, \(P = 0.008\)), and improved survival (median survival, 25.1 vs. 20.3 months; \(P = 0.01\)). However, trastuzumab was complicated by a high rate of cardiac dysfunction (NYHA class III and IV). The incidence of cardiac dysfunction was 8\% in patients treated with a conventional chemotherapy regimen including an anthracycline and increased to 27\% in patients who received trastuzumab concurrently with the same regimen.\(^5\)

The cardiotoxicity of trastuzumab appears to be associated with the ErbB2/gp130-dependent cell-survival pathway.\(^5\) Mice with a ventricular-restricted deletion of ErbB2 display no overt phenotype, but physiologic analysis has revealed the onset of dilated cardiomyopathy with chamber dilation, wall thinning, and decreased contractility. Moreover, cardiomyocytes isolated from these conditional mutants were more susceptible to anthracycline toxicity.\(^5\)

**Fully Human Monoclonal Antibodies**

Several technical advances have finally allowed construction of fully human mAbs. One of these technical breakthroughs is the phage display technology, which isolates fragments with desired specificity from the large and diverse libraries of small human mAb fragment genes (F\(_\text{ab}\) or scFv).\(^5\) The human mAb fragment libraries can be produced from B lymphocytes in various organs of human donors either with (immune library) or without (native library) prior immunization. Small antibody fragments against a wide variety of antigens have been isolated from naive libraries that possess high affinity comparable to those from immune libraries. Application of the phage display technology for human mAb selection is described in Figure 1-9. The affinity of a primary isolate can be further enhanced by mutagene-sis, chain shuffling, or CDR walking, and repeated use of the phage display technology (affinity maturation). The isolated fragment genes are then linked to the genes of the human constant region to create entirely human mAbs. Fully human mAbs can also be generated from transgenic mice carrying human immunoglobulin genes. These technical advances have almost obviated the classic hybridoma technology and the cumbersome humanization process in the production of therapeutic mAbs. Human mAbs are much less immunogenic than chimeric or humanized mAbs, reducing or even eliminating an immune response to these molecules during therapy. A number of fully human mAbs are undergoing preclinical and clinical trials, and the HAHA response has not been reported in humans. Because the technology is relatively new, there are no fully human mAb drugs available as yet for clinical use.

**FUTURE DIRECTIONS**

As noted previously, recombinant protein and antibody therapy represent only a fraction of the current clinical applications of recombinant DNA technology. Although the list of sophisticated recombinant protein drugs will continue to expand, the biotechnology will become intertwined with novel cardiovascular devices that will deliver the protein of interest to an individual organ at a specific time in a variable dosing schedule. Tissue engineering, or cell-based therapy, is an advanced form of *ex vivo* gene therapy, and many successful studies have already been reported in various animal models. The engineering of genes, proteins, cells, and tissues represents different layers of biotechnology, which will evolve complementarily to foster a new era of molecular medicine.

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EDITOR’S CHOICE


CD40 is a biomarker of acute coronary syndromes. Many new cytokines and receptors have been uncovered by the Genome Project, providing a basis for new biomarkers for many cardiovascular indications.


Humanized antibodies to IgE represent a new therapeutic strategy for steroid-dependent asthma.


Humanized antibodies find wide applications in immune related disorders, including psoriasis.


BNP is a sensitive biomarker for the diagnosis of acute decompen-sated heart failure.


Humanized antibodies to VEGF (Avastin) represent a new anti-angiogenic therapy for colon cancer; could find wide usage as adjunctive therapy to other solid tumors.


Three of the acknowledged leaders in immunotherapy via humanized antibodies summarize recent developments in the field.


A humanized antibody (Zevalin) can be engineered to deliver a radioactive payload to tumor cells; “armed” antibodies represent a new direction for immnotherapy.


Humanized antibodies directed against platelet receptors continue to show efficacy in a diverse group of coronary syndromes.