The Immunogenicity of Therapeutic Proteins

The market for therapeutic proteins has grown rapidly over the past decade with new recombinant DNA technology and advances in protein isolation and purification techniques. The worldwide market for therapeutic proteins was $17 billion in 1999, with some of those proteins — such as Epogen (poetin alfa from Amgen) and Intron A (interferon alfa-2b, recombinant, from Schering-Plough) — commanding more than one billion dollars each in annual revenues (1). The pipelines of both large pharmaceutical companies and established biotechnology companies show an increase in antibody and protein-based therapeutics.

Diversity of Protein Therapeutics

Therapeutic proteins are used to treat many diseases, from oncology to inflammation to infectious diseases. The diverse indications for therapeutic proteins perhaps mirror their diverse origins.

Plants and bacteria. Several nonhuman proteins have been developed as therapeutics, including a group of bacterial proteins with potent thrombolytic properties. Those bacterial enzymes, streptokinase and staphylokinase, can target and digest circulatory blood clots such as those that cause myocardial infarctions. They are limited, however, to single administrations because of their tendency to induce immune response (antibody formation) that precludes readministration.

Toxins from both plants and bacteria have been used as cytotoxic agents in the treatment of cancer. The toxins can be specifically targeted, for example by using antibodies (immunotoxins) to kill specific cells such as cancer cells. Clinical studies with ricin (a cytotoxin from the castor bean, Ricinus communis) have shown its selective and potent ability to target and destroy malignant cells (2). However, repeat dosing is prevented by the immunogenicity of the ricin.

Human proteins are being increasingly used and developed as therapeutic molecules.

Many human diseases are caused by the absence or poor expression of a native protein. Acquired disorders (such as insulin-dependent diabetes) and genetic disorders (such as hemophilia A) that were treated with replacement therapy in the past, are now almost exclusively treated with recombinant preparations of the aberrant protein. Yet the recombinant therapies for both diseases carry the risk of immunogenicity.

A strategy of tolerizing the human immune system response to factor VIII has been adopted to circumvent an immunogenic reaction to that molecule. However, making patients tolerate recombinant factor VIII is costly: The estimated cost of immune tolerance induction (ITI) for a pediatric hemophilia patient is close to $1 million (3).

Cytokines are also being developed as therapeutics. Interferon β is used to treat multiple sclerosis, interferon α to treat hepatitis infection, and granulocyte colony-stimulating factor (GCSF) to treat the side effects associated with chemotherapy and HIV. However, the efficacy and patient tolerance of these proteins also is limited by their immunogenicity.

Some patients produce neutralizing antibodies to interferons alpha and beta. Immunogenicity to interferon β1b is prevalent in nearly half of the patients demonstrating antibody production against the therapeutic (4).

Modified proteins. Recently, methods have been developed for reengineering and modifying proteins to enhance their desired properties. Infergen (Interferon alfacon-1 from Amgen) is a consensus interferon molecule for treating hepatitis C. The molecule is constructed from multiple interferon α subtypes rather than a single subtype as routinely used. The rationale for using such a consensus molecule is that desired properties displayed by the individual subtypes can be amalgamated into one engineered molecule.

Immunogenicity is often a barrier to further development of potentially therapeutic proteins. That barrier often remains unknown until late in the development chain. The authors explore various strategies for preventing immunogenicity and for predicting it earlier in the process.
High-throughput technology generates combinatorial proteins that can be screened for enhanced properties and functions. An example is a combinatorial interleukin molecule developed by Parrish-Novak, et al. and isolated by screening an activated human CD3+ T cell cDNA library (5). Functional cloning has identified that molecule as closely related to interleukin 2 (IL-2) and interleukin 15 (IL-15). However, no combinatorial proteins are currently in clinical testing, so that their efficacy and safety have yet to be demonstrated.

**Immunogenicity of Protein Therapeutics**

Proteins of any origin, including human proteins, might elicit an immune response (see Table 1). Immunogenicity can have a number of effects on the therapeutic outcome of a biologic. Immunogenicity can, for instance, reduce the efficacy of a biologic by lowering the molecule’s half-life in the circulatory system because of rapid clearance by immune cells. An immunogenic response can also preclude repeat dosing because readministration would cause a strong immune reaction. Staphylokinase, for instance, can be used only once before a patient produces antibodies against the protein that would manifest as a strong immune reaction against the molecule on subsequent administration (6).

**A development barrier.** The immunogenicity observed with some therapeutic molecules is recognized as a barrier to further development of those molecules for clinical use. The degree of immunogenicity can, however, be affected by the route of administration. Insulin has traditionally been given subcutaneously, which can generate an immune response in some patients at the injection site, so strategies for inhaled insulin and other therapeutic protein molecules are now in development. However, some clinical studies have shown that some protein therapeutics are more immunogenic when inhaled (7).

**Addressing Immunogenicity**

A number of techniques have been developed to try to address the issue of immunogenicity from therapeutic proteins.

**PEGylation** is the covalent attachment of polyethylene glycol (PEG) to lysine molecules on a protein molecule’s surface. It was developed to prolong the molecule’s half-life in the circulatory system and to reduce its immunogenicity. PEG is a nontoxic, highly soluble molecule commonly used in many manufacturing procedures, such as in cosmetic production and the food industry. PEGylation can prevent the binding of the therapeutic to its cellular active site, thus reducing its activity (8).

**Site-specific PEGylation can prevent an immune response.** This table shows the percentage of patients that produce antibodies against the particular protein therapeutic in individual trials.

### Table 1. Several human proteins when administered as therapeutics in clinical trials initiate an immune response. This table shows the percentage of patients that produce antibodies against the particular protein therapeutic in individual trials.

<table>
<thead>
<tr>
<th>Biologic</th>
<th>Indication</th>
<th>Immunogenicitya</th>
<th>Referenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interferon α 2b (Intron A)</td>
<td>cancer, hepatitis</td>
<td>7</td>
<td>18</td>
</tr>
<tr>
<td>Interferon β 1a (Avonex)</td>
<td>multiple hepatitis</td>
<td>24</td>
<td>4</td>
</tr>
<tr>
<td>Interferon β 1b (Betaferon)</td>
<td>multiple sclerosis</td>
<td>45</td>
<td>4</td>
</tr>
<tr>
<td>Interferon alfacon 1 (Infergen)</td>
<td>hepatitis C</td>
<td>11</td>
<td>19</td>
</tr>
<tr>
<td>Factor VIII (Refacto)</td>
<td>hemophilia</td>
<td>30</td>
<td>12</td>
</tr>
<tr>
<td>Insulin sc administration</td>
<td>diabetes</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>Insulin inhaled administration</td>
<td>diabetes</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

*a percentage  
*bReferences, end of article

Site-specific PEGylation can prevent active site blockage, but the surrounding residues may be differentially important in the binding of the therapeutic to its cellular target (9). Clinical trials have demonstrated varying degrees of immunogenicity reduction by these molecules. Some PEGylated molecules have other side effects that limit their efficacy as therapeutics. A PEGylated version of GCSF was withdrawn from clinical trials because of low patient response. In a phase 3 clinical study comparing PEG-Intron with unPEGylated Intron A, PEG-Intron failed to meet the protocol-specified statistical criteria for noninferiority in the treatment of chronic myelogenous leukemia (CML); that is, no observed clinical advantage was found for the PEGylated version of interferon A (10). Those studies highlight some of the difficulties in engineering improved protein therapeutics using pegylation technology.

**Humanizing Therapeutic Proteins**

The humanization process was originally developed for therapeutic antibodies. MURINE antibodies induce antimouse antibodies in humans (the so called HAMA response). Removing as much of the murine sequence as possible and replacing it with human versions was postulated as a method for alleviating the HAMA reaction. That process was successful for some products: Trastuzumab (Herceptin from Genentech, Inc.) for treatment of some breast cancers is approved by the Swiss but has had U.S. approval delayed by FDA. Interferon α in combination with ribavirin is the only currently approved method for the treatment of hepatitis C viral infections. Another PEGylated interferon for the treatment of hepatitis C is peginterferon alfa-2b (PEG-Intron, developed by Enzon as an advanced version of Schering-Plough’s Intron), which was approved by FDA in August 2001.

These PEGylated molecules have displayed varying degrees of improvement over the generic forms of interferon α with improved circulatory half-lives. Circulating levels of PEGAsys were demonstrated to peak at 77 hours compared with four to nine hours for the unPEGylated treatment (8). The increased serum half-life of the PEGylated molecules means patients require fewer doses to maintain the therapeutic levels required in the circulation. However, the PEGylated therapeutics increase the cost of treatment to about $1,000 per month per patient.

**Challenges to PEGylation.** Random attachment of a PEG to the lysine molecules within a protein can allow binding around the active sites of the molecule, reducing its activity and eventually its efficacy. That reduction in activity is addressed by increasing the treatment dosage, which again increases the potential of an immune reaction to those molecules.

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**Identifying and removing T-cell epitopes is an attractive method of reducing immunogenicity and improving the profile of future therapeutic proteins.**

One in other cases, humanized antibodies, such as Campath-1H used for the treatment of rheumatoid arthritis, induce an immune response in 60% of the patients treated (11).

**Exon shuffling.** Another strategy for selecting and redesigning proteins for use as therapeutics with good activity and safety profiles is exon shuffling: directed “evolution” of proteins by altering the position of protein-coding regions within a gene to select for improved characteristics. Exon shuffling of human pharmaceutical proteins should generate libraries in which all the sequences are fully human, but the technology is in its infancy. Protein therapeutics modified using this method have yet to enter the clinic.

**Preventing an Immune Response**

An immune response begins when a protein is taken up by an antigen-presenting cell (APC), such as a dendritic cell. The protein is then proteolytically degraded, and some resulting peptide fragments are bound to major histocompatibility complex (MHC) class II molecules. A small number of those peptides are expressed on the cell surface as a complex with MHC molecules. Those MHC–peptide complexes evoke an effector response when recognized by the antigen-specific receptors on T cells. This triggers a cascade in which some T cells differentiate into helper T cells. The release of cytokines induces differentiation of antigen-specific B cells into antibody-specific plasma cells.

Preventing a T-cell response is critical to preventing an immune response to a therapeutic protein. That is usually achieved by immunosuppression, patient tolerization, or the removal of T-cell epitopes.

**Immunosuppression** involves treatment with agents that nonselectively inhibit the normal function of the immune system’s T cells. This method is widely used to prevent transplant rejection. However, nonselective immunosuppression is limited by the potential repercussions of a nonfunctioning immune system, such as infection. Therefore, immunosuppressive therapy in conjunction with therapeutic proteins is limited to acute, life-threatening conditions only.

**Tolerance** to a therapeutic protein offers a more acceptable means of preventing an immune response. One complication in using factor VIII to treat hemophilia A is the production of inhibitory antibodies to the therapeutic, which is observed in about one-third of all patients (12). The production of antifactor VIII antibodies in those patients is associated with an increased risk of morbidity and requires the selection of alternative hemostatic agents.

One strategy used to overcome the immune reaction to factor VIII is to induce immune tolerance. Daily intravenous administration of large doses of factor VIII along with immunosuppressive agents taken for months to years limits the immune reaction. Therapeutic success varies from patient to patient, ranging between 60–80% (13). The major disadvantage to inducing immune tolerance in hemophilia A patients is cost. It is estimated that inducing tolerance to factor VIII in a pediatric patient costs about $1 million; however, once tolerance is achieved, it lasts the rest of the patient’s life (14).

**T-cell epitope removal** is an increasingly attractive method for preventing an immune reaction. Molecular determinants that drive the immune response against a therapeutic protein are identified and removed. A T-cell epitope is probably best described as a proteolytically generated peptide, which is derived from a protein bound to an MHC class II molecule that is recognized by a T-cell receptor. Rather than identifying peptides recognized by the T-cell receptor, immunogenicity prevention can be achieved by altering the MHC-binding peptides to prevent them from binding to the MHC class II molecules.

**Predicting T-Cell Epitopes**

Identifying T-cell epitopes in a therapeutic protein can aid in the design of an improved molecule with reduced immunogenicity. T-cell epitopes within a target protein can be delineated in vitro by their response to human T-cell mediation. Identifying and eliminating those epitopes can improve a molecule’s safety profile.

Modern human T-cell assays attempt to produce an in vitro surrogate of an in vivo immune response. In one assay format, naïve T cells prepared from human donors are exposed to synthetic peptides and autologous APCs. T-cell proliferation in response to peptide exposure is measured by incorporating a detectable label into the proliferating cells. The number of proliferating cells correlates directly to a peptide’s ability to invoke an immune response. Assay sensitivity is limited, however, so some potentially important T-cell epitopes may be missed. In addition, throughput is hampered by the need to use several replicates for each blood sample and to screen multiple blood samples with different MHC allotypes. Throughput is further reduced if multiple analogues of the T-cell epitopes have to be tested to find analogues that go unrecognized by the T cells.

**In silico prediction of T-cell epitopes.** Knowing the crystal structure of human MHC molecules has aided in formulating epitope prediction matrices that allow the in silico prediction of peptide sequences with the potential to bind to the MHC binding groove and initiate immune cascades. Overlapping peptides within an protein can be analyzed by computational matrices based on rules about binding characteristics. A binding score prediction can then be assigned to a particular peptide sequence.

**Binding motifs.** Research into the prediction of T-cell epitopes has generated antigen processing rules and identified specific motifs implicated in the binding of peptides to MHC molecules. Those binding motifs have aided in designing and constructing computer algorithms for T-cell
epitope prediction. Prediction of peptide motifs involved in MHC class II binding has been aided by the use of large peptide pools to screen for MHC class II binding peptides. The molecules that bind within the peptide binding groove to MHC class II molecules extend out of the cleft because of openings at either end of their structure (15). That open structure allows a more variable peptide length than what can bind within the binding groove of MHC class I molecules. Bacteriophage peptide display libraries then identify particular anchor residues within peptides that act as MHC class II binding motifs (16). Experimental MHC-binding motifs in addition to antigen-processing and putative-processing motifs have encouraged approaches to T-cell epitope prediction in silico (17).

Experimental determination of MHC-binding motifs, antigen processing, and putative processing motifs have led to a valid approach to T-cell epitope prediction in silico. The approach, however, is seldom exhaustive in predicting T-cell epitopes.

**Threading Peptides**

In silico prediction of T-cell epitopes has increased the throughput and feasibility of analyzing multiple protein sequences for predicted binding to MHC class II molecules and subsequent initiation of a T-cell response. Biovax has developed “peptide threading,” an in silico method for predicting T-cell epitopes. The method was initially developed for analyzing side-chain interactions between peptides and MHC class I molecules. We adapted the method to work with class II molecules.

Peptide threading analyzes the binding of every overlapping peptide sequence in a protein to several MHC class II molecules. Using state-of-the art protein-modeling technology, MHC class II allotype models were built based on existing crystal structures for human leukocyte antigen-DR (HLA-DR). In each model, overlapping amino acid sequences of 13 amino acids (13-mers) are threaded, and binding calculations are performed for each peptide based on its fit and ionic and hydrophobic interactions with the MHC class II molecule. In practice, 13-mers with known biologically active T-cell epitopes invariably rank at the top of the range of binding scores, whereas known inactive peptides rank at the bottom. The approach has been validated using protein analysis of known T-cell epitopes, such as tetanus toxin, hepatitis B surface antigen, and influenza hemagglutinin. The system prompts the design of sequences with reduced binding and, therefore, reduced immunogenicity profiles.

**Seeing the Future**

Therapeutic protein molecules are being developed as frontline treatments for a number of diverse indications. The number of proteins in development and on the market will increase dramatically during the next 10 years. Several will elicit immunogenicity in some patients. Identifying and removing T-cell epitopes is an attractive method for reducing immunogenicity and is expected to improve the profile of many future therapeutic proteins.

**References**


