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# Shifting paradigms: biopharmaceuticals versus low molecular weight drugs

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To celebrate and commemorate Prof. Dr. H.E. Junginger's 60th birthday

**Abstract**

Biopharmaceuticals are pharmaceutical products consisting of (glyco)proteins. Nowadays a substantial part of the FDA-approved drugs belong to this class of drugs. Biopharmaceuticals deserve special attention as they have a number of characteristics that set them aside from low molecular weight drugs.

Their activity depends on their complicated shape based on secondary, tertiary and (sometimes) quaternary structures. These structures cannot be fully defined with our present set of analytical techniques and approaches for potency testing. They often are the same as (or closely resemble) endogenous proteins. This means that in safety testing and clinical test programs questions have to be addressed regarding species specific responses, selection of dosing schedules and route of administration, and the possible occurrence of immunogenicity. As the conformational structure of a protein is easily disturbed, formulation and handling of biopharmaceuticals needs special attention in order to optimize the therapeutic effect and minimize adverse reaction, among which immune responses.

The issue of biogenerics is gaining more and more interest and different critical elements in the development of biogenerics are touched upon.

In conclusion, biopharmaceuticals cannot be characterized fully in terms of their structure like low molecular weight drugs. The performance of biopharmaceuticals relies on strict production protocols and close monitoring of their activity in the clinical situation.


*Keywords:* Biopharmaceuticals; Biologicals; Biogenerics; Immunogenicity; PK–PD

*Abbreviations:* ADA, adenosine deaminase; CHO, Chinese hamster ovary; ELISA, enzyme-linked immunosorbent assay; FSH, follicle stimulating hormone; G-CSF, granulocyte colony-stimulating factor; HAMA, human anti mouse antibodies; hGH, human growth hormone; i.m., intramuscular; IEF, iso-electric focusing; IEP, iso-electric point; LHRH, luteinizing hormone releasing hormone; NESP, novel erythropoiesis stimulating protein; PEG-ADA, pegylated adenosine deaminase; PEG-G-CSF, pegylated G-CSF; PK–PD, pharmacokinetics–pharmacodynamics; PLGA, polylactic-coglycolic acid; QCM, quartz crystal microbalance; rhuEPO, recombinant human erythropoeitin; s.c., subcutaneous; SDS–PAGE, sodium dodecylsulfate polyacrylamide-gel electrophoresis; TPO, thrombopoeitin

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## 1. Introduction

Biopharmaceuticals are defined as 'pharmaceutical products consisting of (glyco)proteins and/or nucleic acids' (Schellekens, 2002). In this contribution the term only applies to (glyco)protein products used for therapeutic purposes and vaccines. Biopharmaceuticals still make up only a small part of the total arsenal of medicines that is used by mankind. But, the growth of this category of medicines is much faster than the introduction and growth of 'conventional', low molecular weight medicines. Recent statistics show that the FDA approved 130 biotechnology derived protein medicines and vaccines (Biotechnology Industry Organization Site, 2002), 70% of which were approved in the last 6 years. Currently, over 350 biopharmaceuticals are in clinical trials.

These biopharmaceuticals deserve specific attention by pharmaceutical scientists as they have a number of characteristics that set them aside from low molecular weight drugs. These specific characteristics center around a number of issues listed in Table 1. The issues on this list will be discussed in more detail below.

## 2. Molecular characteristics

Biopharmaceuticals are (glyco)proteins. The building blocks for these molecules are L-amino acids and different sugar molecules. They form three-dimensional structures based on secondary structures (alfa-helices, beta-sheets and random coil areas), tertiary structures (folding of the secondary structures into complicated three-dimensional structures) and in some cases quaternary structures (where different monomers interact). Such special structures do not exist in low molecular weight drug molecules. Different areas in protein drug molecules have different functions. For instance, in a monoclonal IgG type antibody different parts clearly have different functions. A schematic of a monoclonal antibody is depicted in Fig. 1. About 1400 amino acids make up the protein backbone of an IgG antibody. The antigen binding site (responsible for site specific docking on the antigen) is located at the beta-plated N-terminal ends of the Fab part. The sites for complement binding and macrophage interactions are found on the Fc part. *N*-Glycosylation sites are located at the Fc part as well. Small molecules do not possess such sophisticated structures harboring several different functions on one molecule.

Small molecules like aspirin and acetaminophen can be fully described in terms of their molecular structure. For full identification a limited set of analytical assays can be used. Different pharmacopoeial sources describe this in detail. Assays to describe impurity profiles can be found there as well. Typically, 98 to >99% purity is required and suppliers are able

Table 1
Specific characteristics of biopharmaceuticals

| |
|---|
| Molecular characteristics |
| High molecular weight: $M_w$ > a few thousand Daltons (glyco)proteins |
| Pharmacology/therapeutic use |
| Life threatening/severe diseases |
| Safety/clinical testing |
| Species specificity |
| Dosing schedules |
| Route of administration/PK–PD relationship |
| Immunogenicity |
| Formulation |
| Stability |
| Rate controlled delivery |
| Handling |
| Biogenerics: the possibility to launch biogenerics? |

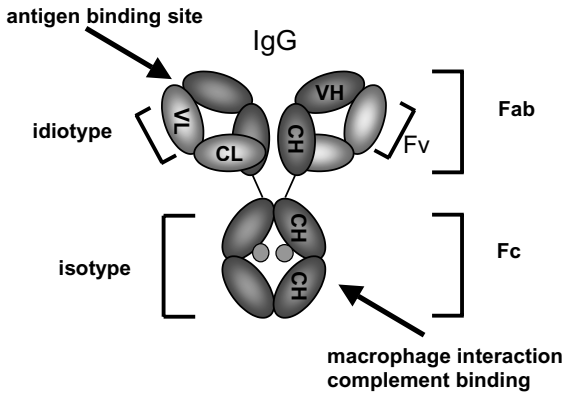


Fig. 1. Antibody structure. Cartoon of IgG antibody structure. Antibodies bind antigen via their variable region (VL and VH). The $CH_2$ and $CH_3$ domains of heavy chains make up the Fc part and determine the biological activity. N-linked glycosylation sites are indicated as small circles. L stands for light chain, H for heavy chain. The small circles represent the glycosylation sites and sugar segments. Adapted from van Dijk and Vidarsson (2002).

to reach that quality level consistently. What about biopharmaceuticals? Later in this contribution we will discuss biopharmaceutical quality in more detail. Here, two differences between biopharmaceuticals and low molecular weight compounds will be dealt with first: these relate to (a) protein production and purity and (b) protein characterization.

### 2.1. Protein purity

The choice of the expression system and growth conditions for the production of biopharmaceuticals is critical. Only a few expression systems are regularly used for the production of biopharmaceuticals, as both manufacturers and authorities are familiar with the specifics of these production systems (e.g. *Escherichia coli* as a prokaryotic and yeast or CHO cells as eukaryotic production cells). General patterns emerged for the optimization of growth conditions and the selection of proper downstream processing protocols to purify the desired protein, removing all host cell-, process- and product-related impurities. Table 2, taken from Kadir and Hamers (2002), lists typical production contaminants both for prokaryotic and eukaryotic production cells that have to be rigorously removed. Here, as with low molecular weight drugs, the ultimate goal is 99+% purity. Meeting that purity level in terms of amino acid sequence may be feasible for smaller proteins (such as insulin or calcitonin: $M_w$ in the range of 5 kDa); sequencing methodology together with chromatographic techniques provide the means to establish those purity levels.

Many biopharmaceuticals like monoclonal antibodies, Factor VIII and FSH are glycoproteins produced in eukaryotic cells. Glycosylation patterns vary, not only between production cell lines but also for one particular production cell line in different growth media and under different growth conditions (e.g. stirring, oxygenation). Fig. 2 shows an IEF diagram of a highly purified monoclonal antibody (adjusted from Kadir and Hamers (2002)). A number of bands are visible, probably reflecting differently sialylated antibody isoforms. Even under well-controlled production conditions these isoforms appear. As isoforms may have different pharmacokinetic profiles and receptor binding affinity, differences in bioactivity can be expected if these patterns are not reproduced within narrow limits. With recombinant FSH, isohormone IEPs fall between 5.49 and 4.27. In vivo activity varies 20-fold going from the highly sialylated (IEP = 4.27) to the less richly sialylated isohormone (IEP = 5.49) (Sam and Peters, 2002). The abundant presence of isohormones may appear difficult to accept. However, one has to realize that FSH protein products with a much lower FSH content containing a lot of non-relevant proteins have been on the market before. Typically, FSH collected from postmenopausal urine had a FSH content of less than 5%. Recombinant proteins, therefore, can offer a huge improvement in pharmaceutical quality.

### 2.2. Product characterization

Establishing the primary amino acid sequence of proteins is relatively easy with state of the art equipment using a combination of trypsin digests, chromatography and mass spectrometry. But what about protein folding? How do we define the secondary, tertiary and, if applicable, quaternary structures of a pharmaceutical protein? To establish proper folding patterns for even one protein structure poses a

Table 2
List of typical production contaminants both for prokaryotic and eukaryotic production cells (taken from Kadir and Hamers (2002))

| Type of nutrient | Example(s) |
|---|---|
| Sugars | Glucose, lactose, sucrose, maltose, dextrins |
| Fat | Fatty acids, triglycerides |
| Water (high quality, sterilized) | Water for injection |
| Amino acids | Glutamine |
| Electrolytes | Calcium, sodium, potassium, phosphate |
| Vitamins | Ascorbic acid, α-tocopherol, thiamine, riboflavine, folic acid, pyridoxin |
| Serum (fetal calf serum, synthetic serum) | Albumin, transferrin |
| Trace minerals | Iron, manganese, copper, cobalt, zinc |
| Hormones | Growth factors |

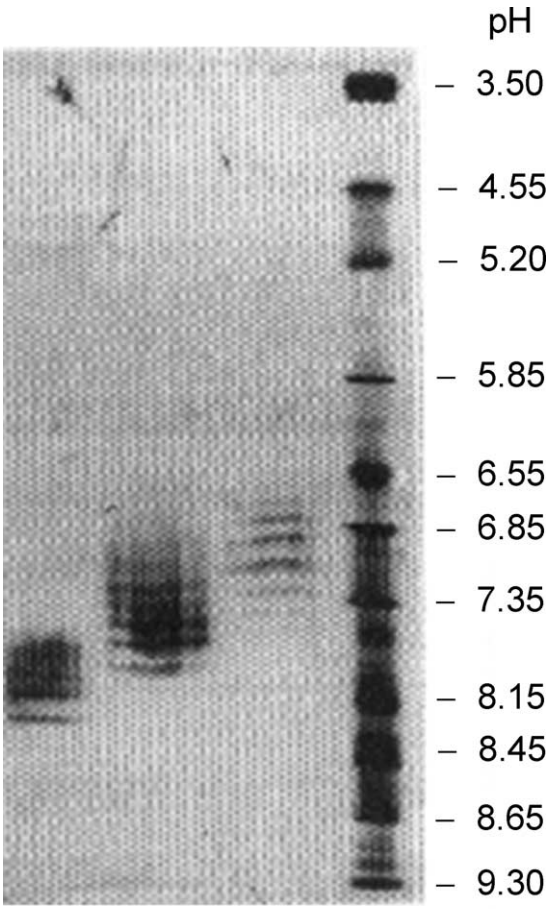


Fig. 2. Silver-stained IEF patterns of three types of purified monoclonal antibodies. The right lane represents marker proteins. Adapted from Jiskoot et al. (1991).

challenge. This challenge increases in magnitude if protein products contain mixtures of proteins, e.g. different isoforms.

Table 3 lists analytical techniques to characterize the structure or function of proteins, but none of these techniques allows us to fully define protein folding patterns. They all provide pieces of information. Combining the different pieces of evidence allows us to build a picture of the protein. But this picture will be incomplete even if all assays are used. One might think that the ultimate test is the potency test. However, potency tests have limited value because of the inherently large variability in outcome of these tests. They also provide little information about immunogenicity.

Table 3
(Analytical) techniques for monitoring protein structure

| |
|---|
| UV absorption |
| Circular dichroism spectroscopy |
| Fourier transform IR |
| Fluorescence spectroscopy |
| NMR spectroscopy |
| Calorimetric approaches |
| Bio-assays |
| Immunochemical assays |
| ELISA |
| Immunoprecipitation |
| Biosensor (SPR, QCM) |
| Potency testing |
| In cell lines |
| In animals |
| Chromatographic techniques |
| RP-HPLC |
| SEC-HPLC |
| Hydrophobic interaction HPLC |
| Ion-exchange HPLC |
| Peptide mapping |
| Electrophoretic techniques |
| SDS–PAGE |
| IEF |
| CZE |
| Field flow fractionaction |
| Ultracentifugation |
| Static and dynamic light scattering |
| Electron microscopy |
| X-ray techniques |
| Mass spectrometry |

Some of these analytical approaches (e.g. X-ray crystallography and NMR) are quite complex and may only be relevant to consider in (early) development stages. Other analytical assays (e.g. SDS–PAGE, ELISA and HPLC analyses) are used routinely in release tests. A full discussion of the pros and cons of all listed assays in Table 3 is beyond the scope of this contribution. Extensive reviews on these analytical approaches can be found, for example in Herron et al. (1995) or Metz et al. (2002).

Some reflection on two selected spectrometric approaches: X-ray diffraction provides information on the full protein, but the information is obtained for a crystallized product in a milieu that is different from the protein environment in the formulated product. Moreover, not all proteins crystallize easily, in particular glycosylated proteins and those that are present in different isoforms offer problems. Crystallization and data analysis can take months to achieve. Therefore,

such approaches would never work as a routine batch release assay. In principle, NMR spectrometric analysis would also be an option to provide full information on protein folding. However, at present structure elucidation of proteins is restricted to structures up to 25 kDa in size. And, for many proteins the required concentrations for NMR analysis exceed those in the formulated material. With NMR, as with X-ray crystallography, the technique lacks sensitivity for minor fractions of (misfolded) impurities and full data analysis takes too much time to be used for batch release decisions.

## 3. Pharmacology/therapeutic use

### 3.1. Life threatening and serious diseases

Table 4 shows a list of biopharmaceuticals marketed in the USA in early 2002 (Evens and Sindelar (2002)). When considering the therapeutic indications, it is clear that these biopharmaceuticals were introduced to treat severe and/or life threatening diseases. The list includes monoclonal antibodies for immune modulation and treatment of cancer; biological response modifiers to stimulate cell growth; hormones such as insulin and hGH; enzymes such as alteplase to remove blood clots; and last but not least, a number of vaccines. The list of indications for the different molecules is expanding rapidly as well. Interestingly, many biopharmaceuticals are endogenous proteins or slightly modified endogenous proteins. Their therapeutic indication can be derived from their mode of action and physiological effects in humans. However, one has to be careful in directly deriving the therapeutic potential of endogenous substances from physiological insights. Upon parenteral administration, severe, dose limiting side effects can be observed because resulting pharmacokinetic profiles are different from those of endogenous proteins. For example, many cytokines work in a paracrine mode: the cytokine is secreted as a mediating molecule by a cell to signal other, neighboring 'target' cells. Upon intravenous injection, however, a cytokine such as IL-2 enters the body far from its target site and may cause severe, dose limiting side effects. It is clear that drug targeting strategies should be considered for all biopharmaceuticals that work in a paracrine mode and which lack intrinsic targeting properties.

Table 4
Marketed biotechnology-produced pharmaceuticals (status early 2002)

| Product | Class[a] |
|---|---|
| Abciximab | MAb |
| Acelluvax | Vaccine |
| Aldesleukin | BRM |
| Alteplase | Enzyme |
| Antihemophilic factor VIII (2)[b] | Enzyme |
| Basiliximab | MAb |
| Daclizumab | MAb |
| Denileukin diftitox | BRM |
| Dnase | Enzyme |
| Entanercept | BRM |
| Epoetin Alfa (6) | HGF |
| Eptifibatide | Enzyme |
| Filgrastim | HGF |
| Factor VII | Enzyme |
| Factor IX | Enzyme |
| Follitropin (2) | Hormone |
| Ganirelix | Peptide |
| Gemtuzumab | MAb |
| Glatiramer | BRM |
| Glucagon | Hormone |
| Growth hormone releasing hormone | Hormone |
| Hepatitis B vaccine (2) | Vaccine |
| Imiglucerase | Enzyme |
| Infliximab | MAb |
| Insulin (3) | Hormone |
| Interferon Alfa (5) | BRM |
| Interferon Alfa con | BRM |
| Interferon Beta (2) | BRM |
| Interferon Gamma | BRM |
| Lenograstim | HGF |
| Lyme disease | Vaccine |
| Molgramostim | HGF |
| Muromonab-CD3 | MAb |
| Nartograstim | HGF |
| Oprelvekin | BRM |
| Panorex | MAb |
| Palivizumab | MAb |
| Peg-Interferon (2) | BRM |
| Polymer-BCNU | Drug delivery |
| Rituximab | MAb |
| Reteplase | Enzyme |
| Sargramostim | HGF |
| Somatropin (6) | Hormone |
| Tenecteplase | Enzyme |
| Tirobifan | Enzyme |
| Trastuzumab | MAb |

Adjusted from Evens and Sindelar, 2002.

[a] Abbreviations include: MAb: monoclonal antibody; HGF: hematopoietic growth factor; BRM: biological response modifier.

[b] The numbers in parentheses indicate the approximate number of products of the same molecule manufactured by different companies.

Endocrine hormones may also act differently when administered parenterally compared to their normal physiological actions. In standard insulin therapy the insulin need of the body of a diabetic cannot be fully mimicked as in a non-diabetic. Maximum and minimum glucose plasma concentration levels cover a wider range in diabetics in spite of the controlled insulin release regimens that are presently available, because of the lack of the 'natural' bio-feedback mechanism. Moreover, the dose is injected s.c. or i.m. in extremities and, consequently, the physiological high liver first pass extraction of pancreatic insulin is not mimicked. This leads to relatively high systemic insulin levels and a disbalance between the intra-hepatic effects and extra-hepatic effects when compared with pancreatic insulin action.

### 3.2. Safety/clinical testing: species specificity

Non-clinical drug safety programs are an intrinsic part of a dossier that is submitted to the regulatory authorities. Over the years a certain routine protocol has been agreed upon. However, for biopharmaceuticals there are reasons to reconsider the use of a standard protocol. First of all, some biopharmaceuticals are species specific. Interferons are an example of a class of biopharmaceuticals well known for its species specificity in terms of pharmacological action. Human interferon does not show the same pharmacological effects as mouse interferon in mice. It may even lack all activity in animals. Secondly, unlike low molecular weight drugs, biopharmaceuticals rarely yield metabolites that are pharmacologically or toxicologically active; they are simply degraded to non-active products. Thirdly, human biopharmaceuticals may readily induce immune reactions in animals. For example, in chronic daily dosing programs antibodies will be neutralizing whatever the effects the biopharmaceutical is expected to have. This induction of antibody formation has no relevance to what may happen in patients. A debatable solution is the use of the murine version of the biopharmaceutical instead of the human version in mice or rats. Or, to use transgenic animals that express the human version of the biopharmaceutical to be tested.

Regularly, clinically relevant discrepancies between the reactions occurring in humans and mice to the administered proteins were reported. Thus, the question may be raised: what test program should be used? Important information is provided by the comparison of the in vitro binding characteristics and functional activity between the biopharmaceutical and its receptor on (relevant) animal and human cells and pharmacokinetic data. In the early 1990s, industry and authorities realized these typical biopharmaceutical-related problems. Safety testing programs on a case-to-case basis were advocated (Bass et al., 1992; Claude, 1992; Cavagnaro, 2002). This led to the ICH document 'Guidance (and not guidelines!) for Industry; S6 Preclinical Safety Evaluation of Biotechnology-Derived Pharmaceuticals' (FDA (1997)). In the introduction of this document the following statement can be found which reflects the spirit of this document:

> 'All three (EU, USA and Japan) regions have adopted a flexible, case-by-case, science-based approach to preclinical safety evaluation needed to support clinical development and marketing authorization. In this rapidly evolving scientific area, there is a need for common understanding and continuing dialogue among the regions. The primary goals of pre-clinical safety evaluation are: (1) to identify an initial safe dose and subsequent dose escalation schemes in humans; (2) to identify potential target organs for toxicity and for the study of whether such toxicity is reversible; and (3) to identify safety parameters for clinical monitoring. Adherence to the principles presented in this document should improve the quality and consistency of the preclinical safety data supporting the development of biopharmaceuticals'.

This document guides the industrial scientist and regulator through the many specific challenges safety testing of biopharmaceuticals offers.

### 3.3. Safety/clinical testing: dosing schedules

Biopharmaceuticals are administered parenterally (except for oral vaccines and those cases where a local action is aimed for). In spite of tireless efforts of a number of groups, such as Junginger and his group (Thanou et al., 2001) oral delivery of proteins and peptides never became a success. Fractions of (intact) peptide and protein absorbed in the GI tract just remain very low and not easy to reproduce. Bell-shaped dose response curves often occur in an-

imal studies with biopharmaceuticals, in particular with cytokines. These curves have been encountered in clinical settings as well (Thomas, 2002). The pharmacological action of cytokines is pleiotropic. These substances influence many different processes in the immune response. Dose increase may thus lead to a full disappearance of the desired effect, because of down-regulation of key receptors, or a signal transduction mechanism where the cells become refractory to subsequent receptor mediated augmentation. Dose finding for drugs with a proven bell-shaped dose response relationship in animals increases the level of uncertainty when starting a clinical trial, in particular when long-term therapeutic effects are defined (e.g. in tumor therapy) and reliable therapeutic markers are difficult to identify (see Talmadge, 1998).

### 3.4. Safety/clinical testing: route of administration/PK–PD relationship

The most common route of administration for biopharmaceuticals is the s.c. injection route. What happens with the protein drug upon s.c. injection? Bioavailability upon s.c. injection varies. It may be close to 100%, but also may be much lower. The fate of the protein depends on a number of factors listed in Table 5, derived from Swartz (2001) and Porter et al. (2000).

Supersaxo et al. (1990) reported that a subcutaneously injected protein can diffuse through the blood endothelial wall entering the blood capillaries at the site of injection, or enter the lymphatic system and reach the blood mainly via the thoracic duct (Supersaxo et al., 1990; Porter et al., 2000). For proteins over 16,000 $M_w$ the lymphatic route of uptake is the primary one. Lower molecular weight proteins are predominantly absorbed in the blood circulation via the local blood capillaries. As lymphatic transport takes time and the protein is exposed to degrading enzymes the absorption rate will be lower and bioavailability is far from complete. Interestingly, there are reports claiming that this lower bioavailability (e.g. 60% for G-CSF, 40% for NESP, rHUEPO) may be compensated for by the prolonged presence of the protein drug in the blood circulation (Kuwabara et al., 1996). But, prolonged presence at the site of injection, as with insulin formulations, may lead to substantial enzymatic degradation of proteins. Insulin resistance in diabetic patients has been described because of high peptidase activity at the site of injection.

Table 5
Factors influencing the pharmacokinetics of biopharmaceuticals upon s.c. injection (derived from Swartz, 2001 and Porter et al., 2000)

| |
|---|
| Molecular weight |
| Animal model |
| Site of s.c. injection |
| Muscular activity |
| Pathological conditions |

Pharmacokinetic profiling of biopharmaceuticals poses specific challenges. Often the protein is $^{125}$I-radiolabeled through its tyrosine or lysine groups to monitor its fate upon injection. However, degradation takes place and the measured radioactivity may no longer exclusively represent the intact protein. Besides, there is always the question whether radiolabeling with iodine changes the pharmacokinetic properties of the protein. Alternatively, immunoassays such as ELISA can be used. Validation of these assays is of prime importance. The issue of cross-reactivity needs to be addressed whenever using immunoassays.

Biopharmaceuticals tend to be highly potent and are dosed in the milligram or even microgram range. Interactions with the vascular walls and their receptors may occur, which have a strong influence on the body disposition of the protein. These interactions are saturable and dose-dependent pharmacokinetic profiles result. Moreover, the PK–PD relationship for biopharmaceuticals is often highly complicated and difficult to describe in simple mathematical models. Recently, Braeckman (2002) reviewed the pharmacokinetics and pharmacodynamics of peptide and protein drugs. An example of a PK–PD relationship (PEG-IL-2) is given in Fig. 3a and b. This example clearly demonstrates that the pharmacodynamic effect of the pleiotropic cytokine PEG-IL-2 is delayed and actually is observed long after the cytokine itself has disappeared from the blood compartment.

### 3.5. Safety/clinical testing: immunogenicity

Biopharmaceuticals are large molecules and therefore prone to elicit an immune response. Factors that play a role in inducing and establishing an immune re-

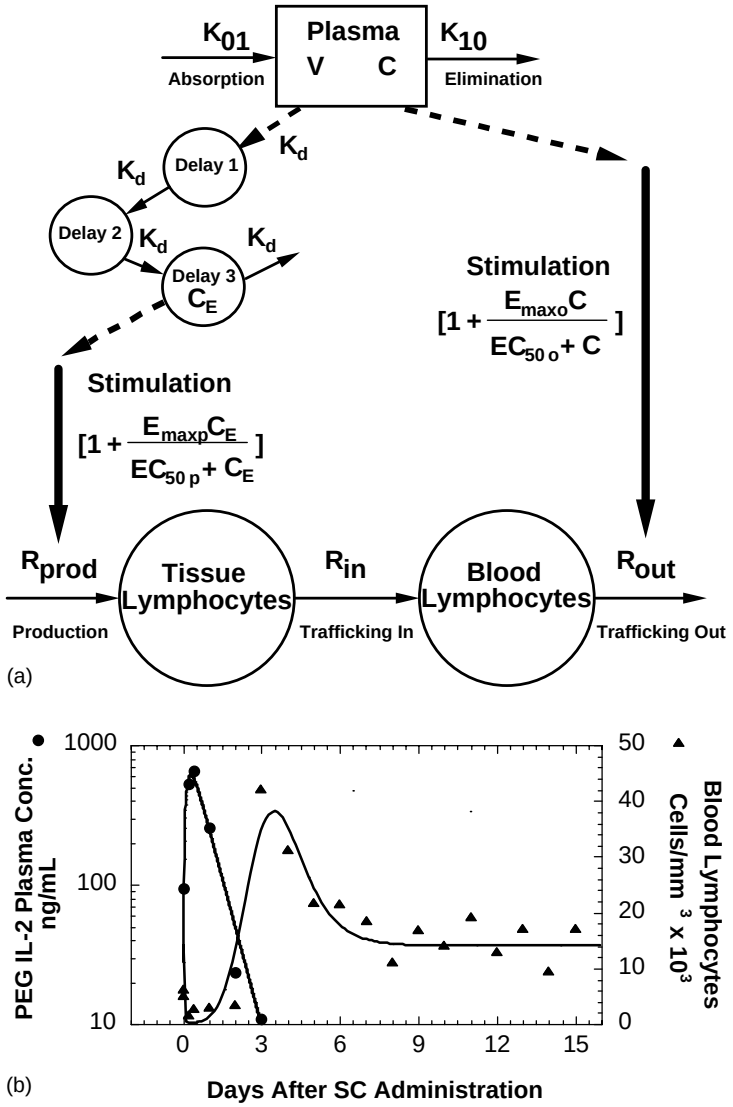


Fig. 3. (a) PK/PD model for changes in blood lymphocytes after s.c. administration of PEG IL-2 in rats. The PK model is a one-compartmental model with first-order absorption (rate constant $K_{01}$) and elimination (rate constant $K_{10}$). PEG IL-2 stimulates the trafficking out of blood and/or catabolism of lymphocytes (first-order rate $R_{out}$) according to an $E_{max}$ model (parameters $E_{maxo}$ and $EC_{50o}$), which is a function of the PEG IL-2 plasma concentration ($C$). The delayed increase of blood lymphocytes is modeled in two consecutive ways: (1) three delay compartments with first-order input and output rates (rate constant $K_d$) resembling distribution and transduction delays; (2) stimulation of lymphocyte production in tissues according to an $E_{max}$ model (parameters $E_{maxp}$ and $EC_{50p}$), which is a function of the effect concentration $C_e$. Tissue lymphocytes traffic into the blood pool (first-order rate $R_{in}$) (from Braeckman, 2002). (b) PEG IL-2 pharmacokinetics and pharmacodynamics (changes in blood lymphocyte count) after subcutaneous administration of 10 MIU/kg in rats, modeled according to the PK–PD model depicted in (a) (from Braeckman, 2002).

Table 6
Factors influencing immune reactions induced by biopharmaceuticals (adapted from Braeckman, 2002 and Schellekens, 2002)

| |
|---|
| Nature of the protein (endogenous/non-endogenous) |
| Contaminants (e.g. host cell material) |
| Route of administration |
| Dose and regimen |
| Formulation |
| Disease and concomitant medication |
| Bioassay design |

sponse are listed in Table 6 (adapted from Schellekens (2002) and Braeckman (2002)). Endogenous proteins are less immunogenic than non-endogenous molecules. That does not mean that endogenous molecules are non-immunogenic per se. Route of administration (immunogenicity s.c. > i.v.), body distribution (e.g. endogenous protein active in paracrine mode versus recombinant protein systemically active), dose (immunogenicity high dose > low dose), duration of dosing (immunogenicity long > short dosing schedules), presence of aggregates and contaminants: all these factors may turn a non-immunogenic molecule into an immunogenic one. Another important factor is the molecule itself. What is claimed to be an endogenous protein may actually not be an endogenous one. Small sequence differences may have been introduced, for instance, for technical reasons (replacement of a cysteine). Or, the endogenous molecule is glycosylated and the recombinant protein is non-glycosylated (as produced in *E. coli*). Or, the glycosylation pattern of the biotech product is different from the endogenous product. These differences may lead to different 'handling patterns' of these biotech molecules in the patient compared to the endogenous molecules, subsequently leading to immune responses. Immunogenic responses occurring with the first generation of therapeutic monoclonal antibodies (HAMA) first led to the introduction of much less immunogenic humanized and, recently, human antibodies, or to the use of only fragments of monoclonal antibodies (e.g. chimeric Fab fragments for abciximab). Another approach to reduce immune responses is to covalently attach polyethylene glycol polymers to the protein (as with PEG-G-CSF, PEG-ADA). This effect is presumably the result of reduced exposure of hydrophobic parts of the protein in the presence of PEG.

Surveying the literature on immunogenicity of recombinant endogenous proteins, makes it clear that immune responses do not occur in all patients. Sometimes only a few percent of the patients develop a response after a year or more of regular exposure. One can only guess what the reason is that not all patients develop antibodies.

Antibody formation can have quite different effects on the therapeutic performance of a biopharmaceutical (Schellekens, 2002; Braeckman, 2002). Reduced therapeutic potency is observed when neutralizing antibodies are formed. Then, the antibody binds to a part of the protein close to or at the receptor binding site. Non-neutralizing antibodies may have an indirect effect on the performance as they may change the pharmacokinetics of the protein drug. Both increases and decreases in therapeutic effect have been observed. A specifically unwanted situation occurs when neutralizing antibodies are formed that cross-react with an endogenous protein and if there is no alternative pathway to substitute for loss of action of that particular endogenous protein. Cases of long-term thrombocytopenia were observed after the use of recombinant thrombopoeitin. Neutralizing antibodies blocked endogenous TPO, which is a crucial factor in the production of platelets.

Formulation design also affects immune response induction. In particular, the presence of aggregates or contaminants (e.g. lipopolysaccharides) has been shown to elicit an immune reaction. During the formulation design stage specific attention should be paid to the stability of the formulation with regard to the prevention of protein aggregation.

Finally, the bioassay used to detect antibodies should be well-validated and internationally standardized, as non-immunogenicity was claimed in the past on the basis of non-validated bioassays (usually ELISAs) (Schellekens, 2002).

In conclusion, antibody formation is highly undesirable and should be avoided as much as possible, but at the present only general rules exist and immunogenicity in clinical situations is highly unpredictable. Several approaches have been developed to predict immunogenicity in man by using animal models. Carefully designed transgenic animals that are immunotolerant to the human protein are presently considered useful predictors, if properly validated (Schellekens, 2002).

## 4. Formulation

Proper protein formulation development is crucial for the optimal therapeutic performance of biopharmaceuticals. As mentioned above, immunogenicity is in some way related to the presence of aggregates and contaminants. As all systemically active proteins are administered parenterally, sterility and non-pyrogenicity are standard requirements for these products. Removal of viruses and other contaminants should be an integral part of the downstream process (Crommelin, 2002). To reach the desired shelf life of two years for biopharmaceuticals, a number of specific challenges have to be met that will be discussed in the following paragraphs.

### *4.1. Stability*

On storage, proteins are exposed to a number of different chemical degradation pathways that are listed in Table 7a. This table also provides a selection of techniques from our analytical 'toolbox' to monitor these degradation processes. Apart from chemical degradation, physical degradation can occur in the form of changes in secondary or tertiary structure, and aggregate formation, which can ultimately lead to precipitation (Table 7b) (Arakawa and Philo, 2002).

Formulation design should be geared to avoid degradation. Proper selection of excipients, physical state and storage conditions are critical to avoid loss of therapeutic value and induction of immune responses (Crommelin, 2002). In principle, the selection of excipients for protein formulations is no different than for low molecular weight formulations. Osmotic agents, buffers and, if necessary, lyoprotectants and antioxidants are found in formulations. Proteins often are highly potent and low doses (milligram or even microgram range) are administered. Low concentrations of anti-adsorbents are added to avoid protein loss or protein denaturation upon contact with the surface of vials, syringes or infusion tubing. Albumin is used (but not everywhere recommended!) as an anti-adsorbent. Low concentrations of surfactants can also be added for this purpose.

If aqueous solutions of the biopharmaceutical are not sufficiently stable, then freeze-drying is an alternative stabilizing technique. But, freeze-drying can readily damage the protein structure. Proper lyoprotection is necessary. Non-reducing sugars can be used for that purpose. Freeze-drying conditions (pressure-,

Table 7a
Common chemical degradation reactions affecting the stability of proteins and methods of analysis (adapted from Arakawa and Philo, 2002)

| | Physical property effected | Method of analysis |
|---|---|---|
| Oxidation | | |
| Cys | | |
| Disulfide | Hydrophobicity | RP-HPLC, SDS–PAGE |
| Intrachain | Size | Size exclusion chromatography |
| Intrachain | Hydrophobicity | Mass spectrometry |
| Met, Trp, Tyr | | |
| Peptide bond | | |
| Hydrolysis | Size | Size exclusion chromatography, SDS–PAGE |
| N to O migration | Hydrophobicity | RP-HPLC |
| Ser, Thr | Chemistry | inactive in Edman reaction |
| α-Carboxy to β-carboxy migration | Hydrophobicity | RP-HPLC |
| Asp, Asn | Chemistry | Inactive in Edman reaction |
| Deamidation | Charge | Ion exchange chromatography |
| Asn, Gln | | |
| Acylation | Charge | Ion exchange chromatography |
| α-Animo group, ε-amino group | | Mass spectrometry |
| Esterification/carboxylation | Charge | Ion exchange chromatography |
| Glu, Asp, C-terminal | | Mass spectrometry |

Table 7b
Common physical reactions affecting the stability of proteins and methods of analysis (adapted from Arakawa and Philo, 2002)

| | | |
|---|---|---|
| Unfolding | Hydrophobicity | RP-HPLC |
| | Secondary structure changes | CD |
| | | FTIR |
| | | Analytical ultracentrifugation |
| Aggregation/ precipitation | Size | SEC |
| | | Light scattering |
| | | Analytical ultracentrifugation |

temperature–time profiles) have to be chosen such that no collapse of the cake occurs during the drying process and the residual water content is low enough to have a glass transition temperature in the freeze dried state exceeding 40 °C.

### 4.2. Rate controlled delivery

Biopharmaceuticals often have to be administered frequently, a number of times daily or a few times per week. To improve patient friendliness several different approaches can be chosen. Modification of the protein is one option. With NESP (darpoeitin) a hypersialylated erythropoeitin is used. At two *N*-glycosylation sites of the protein that do not interact with the receptor carbohydrates with terminal sialic acid groups are attached. This modification increases the $t^{1/2}$ in blood from 9 to 21 h. Similar effects have been described for PEGylation of biopharmaceuticals. PEG-G-CSF (pegfilgrastim, a long circulating form of G-CSF) has recently been approved. Six milligrams pegfilgrastim in one injection proved to be therapeutically equivalent to five daily doses of filgrastim (Physician Package Insert Neulasta® (Pegfilgrastim)). Many years ago, different formulations of insulin were developed that provide controlled release patterns. Zinc or protamine interactions with insulin lead to the preferred blood level of insulin. And a span of duration of action between 6 h for soluble insulin to 28 h for 'ultralente extended insulin zinc suspension' can be achieved. For low molecular weight drugs, release duration may be extended to a few days or to a few weeks by using oil suspensions or solutions (with antipsychotics or hormones). Biodegradable microparticle systems based on polylactic-coglycolic acid (PLGA) have also been described. PLGA in the form of a rods or microspheres has been successfully used for the sustained release (up to six months) of small peptide drugs (e.g. LHRH analogs such as leuprolide). Only if the drug molecule is highly potent

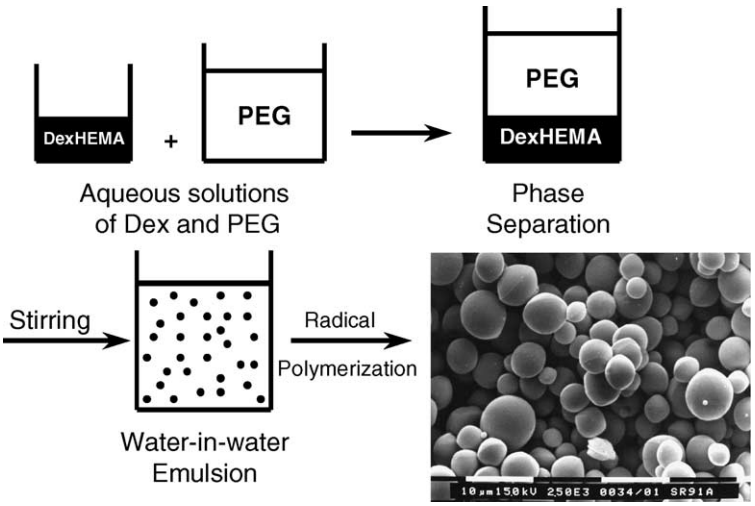


Fig. 4. 'All-water-system' for the formation of protein-loaded microspheres on the basis of biodegradable, cross-linked dextran-hydroxyethyl-metacrylate (dextran-HEMA). Typically, protein yield is >80% and protein loads up to 15% have been described (Stenekes, 2000).

with a daily required dose of <1 mg and if prolonged release for over 1 week is required, does this PLGA technology (or other prolonged release technologies) offer advantages. But, PLGA technology suffers from a number of disadvantages (Crommelin, 2002): (a) strong burst effects are regularly observed (van de Weert et al., 2000a); (b) several reports show that inside the device a substantial pH drop occurs during degradation of the PLGA, affecting the integrity of the PLGA-device associated protein; (c) standard microsphere preparation technology involves a w/o emulsification step, while the protein is dissolved in the aqueous phase. As proteins readily denature at w/o interfaces this process may not be optimal (van de Weert et al., 2000b). There is a clear need for more protein friendly technologies without burst effects. Several hydrogel technologies for controlled release of biopharmaceuticals have been described over the last few years (Hennink and van Nostrum, 2002).

Here we will focus on the 'all-water-hydrogel-microsphere technology' as developed by Hennink et al. (Fig. 4, Stenekes, 2000). When hGH is used, a single injection of hGH gives a similar growth increase as 10 subsequent working days injections covering the same total dose in a dwarf mouse model. The encapsulation efficiency is >90% and the loading capacity up to 15%. No burst release is observed. Some of these controlled release hydrogel technologies have entered clinical test phases, others are in advanced stage of animal testing. Improving patient friendliness of many of the present biopharmaceuticals by reducing the frequency of injection of the chronic dosing schedules through controlling release rates is a challenge taken up by a number of academic groups and delivery companies.

## 5. Handling

During manufacturing and transport to the (hospital) pharmacy the manufacturers should take care of 'Good Transport Practices'. That means that cold chain conditions should be maintained, if so dictated. The receiving pharmacist should do his/her utmost to ensure that these delicate, often expensive, biopharmaceuticals are stored and used as stipulated at the ward by medical staff, or at the patient's home by the patient him/herself. There is a general lack of awareness with regard to the proper use of protein drugs. For example, forced heating and excessive shaking should be avoided at all times as this readily induces denaturation and aggregation, increasing chances for inducing immune reactions. Recently, guidelines for the proper storage and handling of biopharmaceuticals in hospital pharmacies were issued (Crommelin et al., 2003). For conventional, low molecular weight parenterals such strict rules are rather exceptional.

## 6. Generics: the possibility to launch biogenerics(?)

In the production of biopharmaceuticals, seemingly minor changes in production conditions may lead to subtle changes in molecular structure of the protein. Folding might be different, or glycosylation patterns might change. For relatively small proteins such as insulin, equivalence might be established using a set of assays from our 'analytical toolbox' (Table 3): this is the category of the 'well characterized biopharmaceutical'. But, for larger proteins, it is almost impossible to guarantee full equivalence of the protein product characteristics in the clinic, including equivalent efficacy and safety (cf. immune response), when using the full content of our present range of sophisticated analytical techniques. The maximum degree of product equivalency can be reached by keeping all conditions in the manufacturing process constant from batch to batch, starting with the working cell bank, fermentation conditions, downstream processing, filling, finishing and storage conditions of the biopharmaceutical. Besides, a set of analytical techniques, including bioassays, should be run to further support equivalence claims. Over the years, the FDA has developed a 'Guidance for Industry. Changes to an Approved Application for Specified Biotechnology and Specified Synthetic Biological Products'. For minor changes in production protocols a limited comparability program has to be run, but for major changes additional clinical tests have to be performed to establish comparability. Considering the restrictions regarding 'in house' changes of production processes, it is very unlikely that generic versions of biopharmaceuticals will enter the market along the

same regulatory pathways as low molecular weight generic products do. Actually, there are already a number of hGH products on the market developed independently by different companies (e.g. Marian, 2002). Considering the above analysis, the question can be raised whether they ever will be launched for the more complicated, 'not-so-well-defined', biopharmaceuticals.

## 7. Conclusions

Biopharmaceuticals are very different from low molecular weight drugs. The complicated protein production processes and structures ask for a paradigm shift in thinking compared to low molecular weight drugs. No absolute description of drug and drug product is possible with these materials. Our analytical toolbox content and bioassays, including animal testing, are important in ensuring drug quality, efficacy and safety issues in the development phase. But, the biopharmaceuticals rely critically on strict production protocols, clinical expertise and performance monitoring in the clinical situation.

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