DOI 10.1002/biot.200800241 Biotechnol. J. 2009, 4, 186–201

Review

Emerging trends in plasma-free manufacturing of recombinant protein therapeutics expressed in mammalian cells

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Mammalian cells are the expression system of choice for therapeutic proteins, especially those requiring complex post-translational modifications. Traditionally, these cells are grown in medium supplemented with serum and other animal- or human-derived components to support viability and productivity. Such proteins are also typically added as excipients and stabilizers in the final drug formulation. However, the transmission of hepatitis B in the 1970s and of hepatitis C and HIV in the 1980s through plasma-derived factor VIII concentrates had catastrophic consequences for hemophilia patients. Thus, due to regulatory concerns about the inherent potential for transmission of infectious agents as well as the heterogeneity and lack of reliability of the serum supply, a trend has emerged to eliminate the use of plasma-derived additives in the production and formulation of recombinant protein therapeutics. This practice began with products used in the treatment of hemophilia and is progressively expanding throughout the entire industry. The plasma-free method of producing recombinant therapeutics is accomplished by the use of both cell culture media and final product formulations that do not contain animal- or human-derived additives. A number of recombinant therapeutic proteins for the treatment of several different diseases have been produced by plasma-free processes, with the objective of improving safety by eliminating blood-borne pathogens or by reducing immunogenicity. This review describes the factors that drove the development of plasma-free protein therapeutics and provides examples of advances in manufacturing that have made possible the removal of human and animal-derived products from all steps of recombinant protein production.

Received 30 September 2008
Revised 3 December 2008
Accepted 5 December 2008

Keywords: Serum-free · Animal-free · Emerging pathogen · Recombinant therapeutic protein · Biopharmaceutical manufacturing

1 Introduction

Recombinant therapeutic proteins can be produced in microorganisms, plant cell cultures, insect and mammalian cell lines, or transgenic animals. Over 165 biopharmaceutical products have been approved globally [1] and the vast majority of these are proteins. Protein therapeutics were initially ex-

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Abbreviations: **BSE**, bovine spongiform encephalopathy; **CBER**, Center for Biologics Evaluation and Research; **CDC**, Centers for Disease Control and Prevention; **CHO**, Chinese hamster ovary; **FDA**, Food and Drug Administration; **FBS**, fetal bovine serum; **FDA**, Food and Drug Administration; **FVIII**, coagulation factor VIII; **HBV**, hepatitis B virus; **HCV**, hepatitis C virus; **hGH**, human growth hormone; **HIV**, human immunodeficiency virus; **HSA**, human serum albumin; **mAb**, monoclonal antibody; **MASAC**, Medical and Scientific Advisory Council of the National Hemophilia Foundation; **MDCK**, Madin-Darby canine kidney; **NAT**, nucleic acid amplification testing; **NLE**, non-lipid enveloped; **PEG**, polyethylene glycol; **PF**, plasma-free; **rhIFN**, recombinant human interferon; **RNF**, Rebif New Formulation; **SARS**, severe acute respiratory syndrome; **TNF**, tumor necrosis factor; **UNAIDS**, Joint United Nations Programme on HIV/AIDS; **vCJD**, variant Creutzfeldt-Jakob disease; **WHO**, World Health Organization; **WNV**, West Nile virus

tracted from the tissues or blood of humans or other animals and in some cases, human plasma-derived products still remain the only therapeutic option (*e.g.*, intravenous immunoglobulin). A major benefit of recombinant proteins is that they serve as a platform for the development of more advanced products that are engineered for an improved therapeutic profile, such as enhanced safety, lower immunogenicity, increased half-life and improved bioavailability.

A number of recombinant therapeutic proteins, including recombinant human insulin, are produced in microbial expression systems established using bacteria or yeast. However, the production of larger, more complex proteins often requires extensive post-translational modifications, particularly glycosylation. Most microbial systems cannot perform all of the modifications necessary for proper protein structure and function. Instead, mammalian expression systems have been the traditional approach for the large-scale manufacture of therapeutic proteins which require these critical post-translational modifications. An additional advantage of these mammalian cell systems is that the recombinant proteins are secreted into the media in their natural form. *E. coli*-expressed proteins, on the other hand, mostly accumulate in a highly denatured form within the cell as inclusion bodies and require renaturation later in the manufacturing process [2]. About 60-70% of all recombinant protein therapeutics are produced in mammalian cells, primarily Chinese hamster ovary (CHO) cells [3]. CHO cells have many characteristics that have made them the most widely used mammalian host system. In addition to ease of manipulation and other characteristics preferred for large-scale production of proteins, proteins produced in CHO cells have a proven safety profile in humans and these proteins also have glycosylation patterns similar to that of human proteins [4]. Advances in modulating the glycosylation patterns of some yeast strains (*e.g., P. pastoris*) may lead to an alternative, non-mammalian cell system in the future [5] but currently, mammalian cells remain the primary vehicle for large-scale production of recombinant therapeutic glycoproteins.

Hemophilia A is an X-linked coagulation disorder resulting from mutations in the coagulation factor VIII (FVIII) gene. Patients with severe hemophilia rely on exogenous factor VIII therapy to prevent or counter the debilitating and potentially life-threatening consequences of prolonged bleeding [6]. Driven primarily by the need for improved safety [7], factor VIII replacement therapy has evolved from the initial use of whole blood, citrated plasma and cryoprecipitate. The first step in this evolution was the switch to plasma-derived purified FVIII concentrates in the 1970s, followed by recombinant FVIII concentrates starting in 1992, and finally to animal- and human-plasma free recombinant FVIII starting in 2003 (Fig. 1). In the absence of a cure for hemophilia, the development of plasma-free (PF) recombinant FVIII eliminated the risk of blood-borne infections during lifelong therapy.

Additives in biopharmaceutical manufacturing can be derived from a variety of human or animal sources, including blood, milk, bones, hides, tendons, hair, skin, or pancreas (Fig. 2). The main threat, however, is associated with the use of blood or serum-derived proteins. Traditionally, cells are grown in a serum-based medium rich in growth factors and supplements, without which therapeutic proteins could not be produced on a commercial scale. Serum, however, presents many challenges for biopharmaceutical manufacturing. Viral outbreaks and the mad cow disease epidemic highlighted the risk of pathogen transmission through serum, and concerns arose over the safety of recombinant proteins produced by cells grown in the

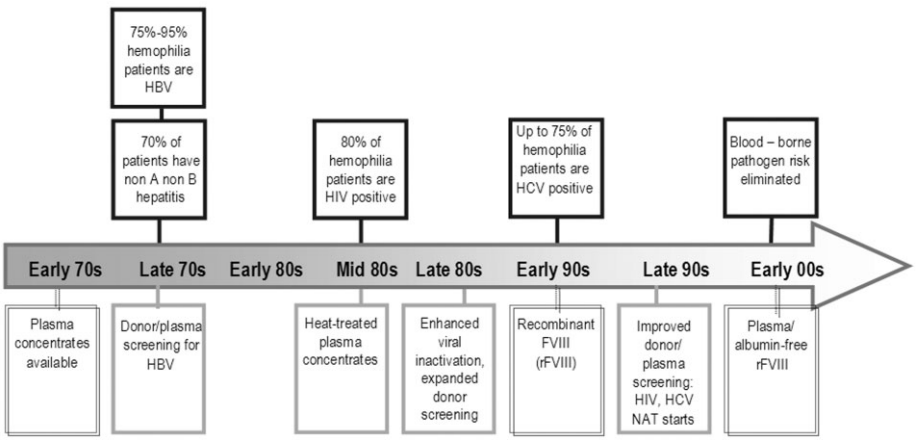

Figure 1. Evolution of safety measures in factor VIII replacement therapy. Boxes above the arrow highlight pathogens that were transmitted to hemophilia patients through blood-derived additives. Boxes below the arrow represent the processing steps that enabled higher purity products to be developed, leading to plasma-free recombinant factor VIII. NAT, nucleic acid amplification testing.

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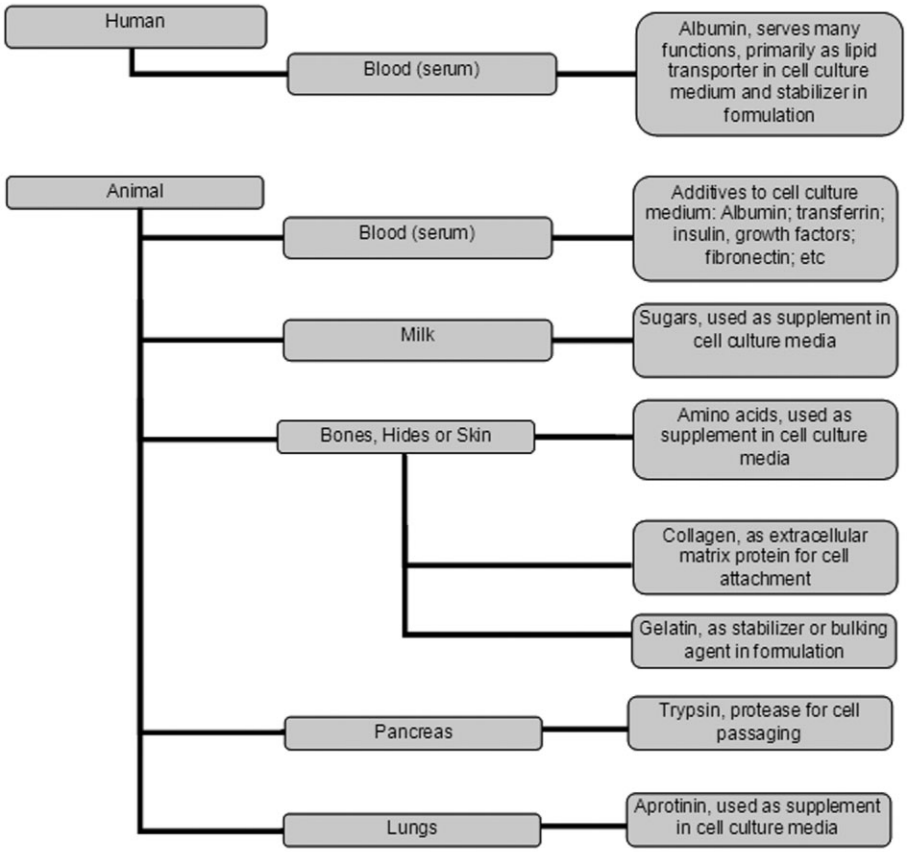


Figure 2. Potential sources of human or animal components that have been used in the manufacturing of recombinant therapeutic proteins. These additives can be derived from a number of tissues and have been used in cell culture medium during production and/or downstream formulation.

presence of ingredients sourced from humans or animals. In addition, the recombinant protein production process can be encumbered by the requirement for serum, due to serum's high protein content and variability, and the inclusion of serum can potentially increase the immunogenicity of certain products. This review will discuss the drivers behind the development of the plasma-free process, the response by regulatory and physicians' organizations, and the advances that have enabled the elimination of serum-derived additives from the manufacturing of recombinant therapeutic proteins.

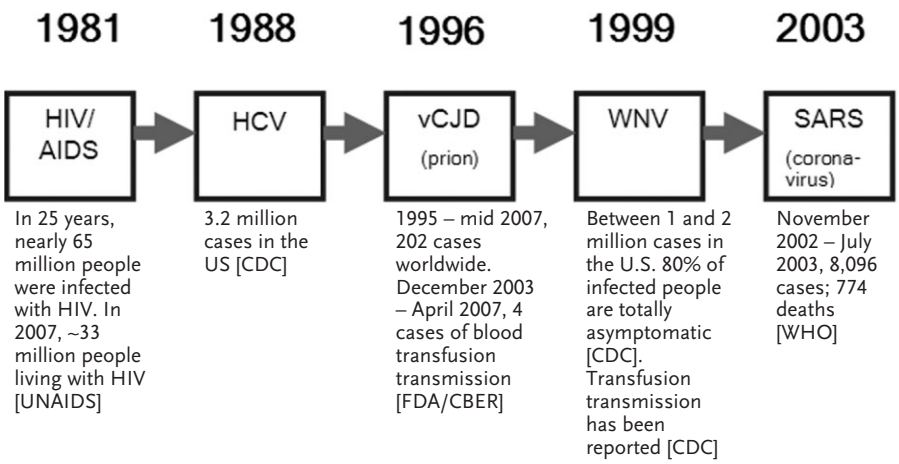


Figure 3. Examples of the most commonly known emerging pathogens from the last three decades. HIV, HCV, vCJD, and WNV have been shown to exhibit blood-borne transmission.

2 Drivers for the development of a PF manufacturing process

2.1 Threat of infectious diseases: Lessons from the past and outlook for the future

Serum and its components are routinely used in cell culture media to support the growth of cell lines. Serum components such as albumin and gelatin are commonly used as stabilizers in the formulation of recombinant therapeutic proteins (Fig. 2). The past several decades, however, have brought to light the serious risks associated with the use of animal or human components in the manufacture of therapeutics, most notably the transmission of infectious agents (Fig. 3). This risk is amplified when human and/or animal-derived materials are used at multiple steps in the manufacturing process, or when a treatment modality requires repeated administrations of such a product.

2.2 Risk of virus transmission

Many emerging blood-borne infectious agents are characterized by long-lasting, silent carrier states in which the pathogen is present in the circulation without causing obvious or noticeable symptoms [8]. Blood or plasma collected during this asymptomatic latent phase can be highly infectious. This is exemplified by human immunodeficiency virus (HIV) which has infected nearly 65 million people worldwide. An estimated 33 million people are currently infected with HIV, yet the vast majority of these individuals are unaware of their HIV^+ status [http://www.unaids.org/en/KnowledgeCentre/HIVData/GlobalReport/2008/].

The coronavirus which is responsible for severe acute respiratory syndrome (SARS) is another example of an emerging human pathogen resulting from zoonotic transmission [9]. Unlike HIV, this coronavirus caused a severe but short epidemic following adaptation to its new human host. Many epidemiologists predict similar types of outbreaks from emerging viruses in the future, with the greatest threat to biopharmaceutical manufacturing being the non-lipid enveloped (NLE) viruses such as circoviruses. Circoviruses, which include torque-tenovirus (TTV) and torque-tenominivirus (TTMV), are the smallest of all known mammalian NLE viruses and they cannot be eliminated by nanofiltration [10].

Historically, the risk of pathogen transmission was of particular concern in the manufacture of blood-derived coagulation factors. In the early 1980s, plasma-derived factor-replacement products used to treat hemophilia were found to be contaminated with HIV and hepatitis B and C viruses (Fig. 1). By 1984, up to 78% of US patients with hemophilia were infected with HIV and 74-90% were infected with HCV [11]. Consequently, the life expectancy for patients with hemophilia in the US decreased from 68 years in the 1970s to 49 years in the 1980s.

Subsequently, new donor screening methods as well as virus- inactivation and removal measures have been adopted. These methods have further improved the safety of plasma-derived or plasma-containing products such that no new cases of HIV, HBV, or HCV transmission from plasma or recombinant therapies have been reported in the US since 1997 [www.hemophilia.org]. However, solvent/detergent, which is the most common virus inactivation technique used in the preparation of plasma-derived products, is mainly effective against lipid-enveloped viruses, such as HIV, HBV, HCV, HTLV (human T-cell lymphotropic virus) and WNV (West Nile virus). NLE viruses, such as parvoviruses, enteroviruses and circoviruses, are resistant to inactivation via solvent/detergent treatment and to some degree against heat and radiation as well [12]. Parvovirus B19 (B19V) and two

Table 1. Currently available recombinant factor VIII concentrates. Shaded boxes denote the presence of human-or animal-derived serum components

	Components in cell culture medium	Primary stabilizer in formulation
First Generation		
Recombinate[a)]	Bovine serum proteins	Human Serum Albumin
Second Generation		
ReFacto[b)]	Human Serum Albumin	Sucrose
Kogenate FS[a)]/Helixate FS[a), c)]	HPPS (Human Plasma Protein Solution)	Sucrose
Third Generation		
Advate[a)]	Non human- or animal-derived	Trehalose
Xyntha[b), d)]	Non human- or animal-derived	Sucrose

[a)] Full-length FVIII.
[b)] B-domain deleted FVIII.
[c)] FS in the US, NexGen in Europe.
[d)] US only. Contains *E. coli*-expressed insulin.

genotypes of parvovirus PARV4, have been found as contaminants in plasma-derived factor VIII concentrates [13, 14]. Thus, the risk of viral infection remains when plasma-derived and plasma-containing recombinant FVIII products are used [15, 16].

The cloning of the FVIII gene in 1984 [17, 18] led to the development of the first recombinant FVIII therapy (Recombinate, Baxter), which was initially licensed in the US in 1992. Recombinant FVIII products are now classified into three generations (as defined by MASAC, the Medical and Scientific Advisory Council of the National Hemophilia Foundation) based on the degree of elimination of plasma and albumin from production and/or formulation steps, as outlined in Table 1 [MASAC #182]. First generation products use plasma and/or albumin during both the cell culture process and the final formulation steps, while 2nd generation products use serum proteins during the cell culture process only. These early recombinant products were effective in reducing the risk of pathogen transmission but the presence of serum components was still a concern. Indeed, one first generation recombinant FVIII product containing human serum albumin (Kogenate, Bayer) was suggested as a source of B19V transmission [19]. Moreover, the presence of human or animal proteins in therapeutic products potentially puts patients at risk of contracting novel infections, including those caused by not-yet detectable pathogens. These concerns prompted MASAC to issue recommendation #106: "Manufacturers of the recombinant products are strongly encouraged to avoid using human and animal proteins in manufacturing their products." Only by completely removing plasma proteins from the cell culture and final formulation processes could the risk of infectious disease transmission from recombinant products be totally eliminated. Such plasma-free recombinant proteins were classified as third generation products.

The first recombinant FVIII product that fit these criteria, (Advate, Baxter), became available in the US in 2003 and in the EU in 2004. Advate is produced in CHO cells that were adapted to grow in a medium free of human- and animal-derived additives. Specifically, the serum- and protein-free synthetic minimal medium used for producing Advate contains ultrafiltered soy bean peptides with an average molecular weight of < 500 Daltons, along with sugars, salts and other components which are added to further nourish the CHO cells. The FVIII is then purified through a series of chromatography steps, including an immunoaffinity chromatography process utilizing a monoclonal antibody produced from hybridoma cells grown for more than 30 generations in a plasma-free environment. Advate is then stabilized with trehalose [20]. Therefore, since Advate is never exposed to plasma, the risk of transmitting known and emerging blood-borne pathogens is eliminated. Current manufacturing processes address disease threats that have endangered the blood supply in the past, but there are others for which donated plasma is not routinely screened [12]. For example, human herpesvirus 8, the causative agent for Kaposi's sarcoma, has been shown to be transmitted through blood and blood products [21, 22]. Although the lipid membranes of enveloped viruses generally render them susceptible to inactivation by combinations of solvents and detergents, the transmission of lipid-enveloped WNV via blood transfusion [http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5313a1.htm] still raises new concerns about the safety of the blood supply. Therefore, due to the fact that solvent/detergent and nanofiltration techniques are not 100% efficient against all pathogens, plasma components need to be completely removed from the manufacturing process in order to eliminate the risk of pathogen transmission through blood compo-nents.

2.3 Risk of transmissible spongiform encephalopathies (TSEs)

Prions are self-replicating infectious proteins that cause currently untreatable and fatal neurodegenerative disorders. The detection of a new strain of prions in humans in the 1990s prompted significant changes in regulatory requirements for biopharmaceutical manufacturing and in public health policy. Consumption of meat contaminated with bovine spongiform encephalopathy (BSE) caused an outbreak of variant Creutzfeldt-Jacob disease (vCJD) in the United Kingdom in 1996 [23, 24]. As of September 2008, 206 primary cases of vCJD had been reported in 11 countries, including the UK, US and France [http://www.cjd.ed.ac.uk/vcjdworld.htm]. Unlike cases of classic CJD, the prion that causes vCJD is found in higher concentrations in lymphoid tissues, and therefore presents a potential threat of transmission through blood and plasma [25].

Indeed, four transfusion-transmitted cases of vCJD have been reported [http://www.hpa.org.uk/infections/topics_az/cjd/vCJDBloodDonors.htm, 26, 27]. In each confirmed case, the patient had received red blood cell products from donors who later developed vCJD. The latest case reported by the UK Health Protection Agency described an individual who was diagnosed with vCJD nine years after receiving a blood transfusion from an asympto-

matically infected donor. None of the four cases of transfusion-transmitted vCJD transmission have been associated with plasma-derived factor concentrates. No cases of vCJD transmission via blood transfusion have been reported in the US. However, the potential for vCJD transmission via plasma products cannot be excluded yet [28]. A nine year study in sheep [29] demonstrated that two different TSE agents (BSE and scrapie) have unexpectedly high rates of transmission through blood transfusion, with relatively short and consistent incubation periods. These experiments suggest that the infectivity titers in the blood are quite high, and that blood transfusion represents an efficient route of transmission for prions.

Adding to the concerns, prions are highly resistant to physical/chemical inactivation and virus-removal methods do not specifically target them [30]. Additionally, there is no way to detect prions in plasma donors who may have early stage, presymptomatic infections. Epidemiologic models generated from vCJD patients predict protracted preclinical disease states and incubation times that may exceed 50 years in individuals with certain genotypes [31].

Iatrogenic transmission of prions has occurred in patients who received human-derived pituitary hormones such as human growth hormone (hGH) and gonadotropins [32, 33]. CJD was transmitted to over 160 recipients of cadaveric pituitary hGH [33-36] prior to its withdrawal in most countries in 1985. Similarly, cadaveric pituitary-derived gonadotropins used to treat infertile women prior to 1985 were associated with iatrogenic transmission of CJD [32, 37, 38]. Cadaveric pituitary hGH has since been replaced with microbially-produced recombinant GH and recombinant gonadotropins (CHO expressed) such as rhFSH have been in use since the the mid-1990s.

vCJD is caused by a human-adapted form of the prion responsible for BSE. Unlike other prions, the adapted BSE prion has infected multiple species in addition to humans [39], which suggests the possibility that additional emerging diseases may result from transmission of prions not currently known to infect humans [40]. One such transmissible prion disease is Chronic Wasting Disease (CWD),which affects elk and deer and potentially poses new challenges to both animal and human health [41, 42]. Removal of all animal- or human-derived raw materials or additives from biopharmaceutical manufacturing eliminates the threat of infectivity by known and other, yet unknown prions.

2.4 Unreliability and heterogeneity of serum and raw source materials

With the discovery of any new infectious agent in humans, the safety of the blood supply is reassessed, often resulting in expanded donor deferral or further screening recommendations. The increased exclusion of donors threatens the adequacy of the blood supply and its derivatives, increasing the risk of shortages [43]. This impacts the availability of serum and of plasma- or serum-dependent products and additives.

To further compound the issue, all sera used in the manufacture of biopharmaceuticals is an undefined mixture of individual donor sera, and therefore varies in composition from one lot to the next [44]. Consequently, products extracted from different pools of sera are also heterogeneous. Such variability imposes the requirement for additional quality assurance steps in the manufacturing process, which can negatively impact productivity and operating costs [44, 45]. Since the manufacture of recombinant plasma-free products does not require plasma-derived ingredients, PF technology not only resolves the safety issues but also enables manufacturers to deliver large quantities of these proteins independent of the global plasma supply.

Concerns about safety, process consistency, product performance, regulatory compliance and reliability of supply prompted many biologics manufacturers to progress from the use of serum to the use of serum-free cell culture media. The next step in the progression was to use animal-free media and ultimately the switch was made to a protein-free, completely synthetic, chemically defined media. Development of serum-free media is generally difficult and must be individually tailored for each process and cell line. In fact, even different clones of a given cell line, like CHO cells, may require different formulations for optimal growth [46]. Additionally, the shift away from serum often comes with the trade-off of reduced yields. Various supplements are currently used to mimic the effects of serum in promoting cell viability and optimal productivity, including protein hydrolysates derived from yeast, soy and wheat [47]. These hydrolysates contain amino acids, peptides, carbohydrates, vitamins and essential elements, and are ultrafiltrated to remove endotoxin, residual proteins and contaminants, and to ensure a consistent average molecular weight.

The three most relevant components in serum are albumin, insulin and transferrin. Traditionally, bovine serum albumin (BSA) has been added to serum-free medium as a lipid transport system, a source of intermediary metabolites, and as a pro-

tective agent against shear forces and oxidation [48, 49]. Recombinant animal-free human albumin is now commercially available for cell culture, as is recombinant human insulin (produced in *E. coli* and yeast), and *E. coli* expressed recombinant human insulin-like growth factor. Similarly, yeast-derived, animal-free recombinant human transferrin is on the market [49]. These supplements are among a growing list of animal-free reagents, supplements and enzymes that are being commercialized to support plasma-free mammalian cell culture.

Other advances have been made in generating cell lines that are less dependent on sera-derived components. Examples include CHO cells engineered to produce their own transferrin and/or insulin-like growth factor-I [50] and CHO cells that co-express von Willebrand factor (vWF) along with FVIII. vWF serves as a carrier for FVIII in plasma and is thought to have a stabilizing effect on FVIII. The co-expression of vWF with FVIII leads to increased accumulation of FVIII activity in the absence of serum in the growth medium [51].

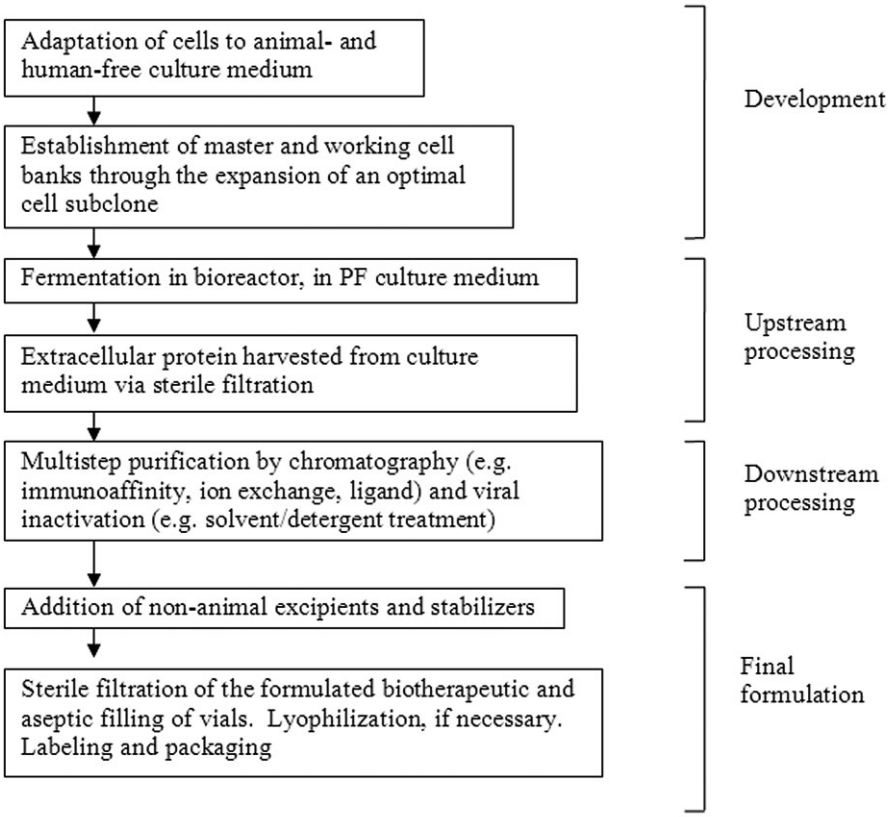
Beyond the production phase, serum-derived components have traditionally been used to address challenges associated with the formulation of protein therapeutics. Recombinant therapeutic proteins are required to be stable, with a significant shelf life and good bioavailability. These desired characteristics present a challenge for product formulation because therapeutic proteins have complex tertiary structures and are often up to 1000 times larger than standard small molecule drugs. Therefore, because proteins are often sensitive to a wide range of factors such as temperature, pH, surface adsorption and salt concentration, they are typically formulated with a mixture of excipients to ensure stability. These excipients include sugars, polyols, amino acids, polymers and often, serum albumin. In most cases, the final material must then be lyophilized to avoid loss of activity during storage.

For liquid formulations, it is frequently necessary to prevent protein denaturation at the air-liquid interface, and non-ionic detergents, such as polysorbate, are typically used for that purpose. In addition, it is often the case that the recombinant protein is required in very small therapeutic doses and can be denatured by surface adsorption to glass vials. To prevent this issue, bulking agents like carbohydrates (*e.g.,* sucrose), or proteins such as human serum albumin (HSA) or animal-derived gelatin are added in excess of the active protein. As a formulation excipient, HSA has an excellent stability and an acceptable safety profile [52, 53], and along with gelatin, was traditionally among the most commonly used excipients. However, as is the case with all plasma-derived products, the risk of pathogen transmission could not be eliminated. Additionally, HSA exhibits structural heterogeneity and batch-to-batch variability; its pharmacopoeial purity requirement is only ≥ 96%, with the undesired components being a mixture of polymers of HSA and other contaminating plasma proteins remaining from the purification process [54]. Such concerns prompted manufacturers to switch to sugar-based final formulations and to develop a recombinant, plasma-free albumin produced in *Saccharomyces cerevisiae* for use as a stabilizer in biopharmaceutical manufacturing [55]. Similarly, *Pichia pastoris* yeast was engineered to produce recombinant animal-free gelatin, which presents a reduced risk of allergic responses compared to tissue-derived substances [56, 57].

PF manufacturing requires the elimination of plasma derivatives in every step of the process (Fig. 4), and proper post-production testing to ensure that the product has not been compromised due to stress on the protein-producing cells, which can lead to an aberrant expression profile. The individual steps of the plasma-free production process include: (i) Development – Selection of a cell line, transfection of cells and establishment of viable cell banks that can yield high protein output in serum-free medium (SFM), as well as retain viability and productivity after cryopreservation under serum-free conditions. This requires an extensive process of weaning and adaptation of the cells; (ii) Upstream processing – Production of a protein that is stable in animal-free cell culture medium; (iii) Downstream processing – Purification of the protein without the addition of plasma proteins; (iv) Final formulation – Formulation of the product without albumin or animal-derived additives; (v) Testing – Rigorous testing to assure the safety and efficacy of the PF product in a pre- and post-marketing setting and to ensure that conformation/integrity of the molecule has not been compromised.

2.5 Improving clinical outcomes through plasma-free therapeutics

Most recombinant human proteins have been shown to be immunogenic [58], although the antibody responses they generate are generally variable and of low titer. However, these antibodies can inhibit drug efficacy and cause life-threatening complications when directed against either endogenous self-proteins or exogenous therapeutic proteins [59]. The production of antibodies against recombinant proteins is influenced by several factors, including structural properties of the protein,



a) Not represented are the frequent testing steps for microbial sterility and tests for the integrity of the structure and function of the drug substance

Figure 4. General scheme for commercial development and production of a plasma-free recombinant therapeutic protein. In every step of the manufacturing process, animal- or human-derived additives are replaced by plant-based or synthetic molecules[a].

formulation, dose, route of administration, presence of impurities and the formation of aggregates [60]. For example, high molecular weight complexes formed between rhIFN-α2 and HSA have been linked to the formation of neutralizing antibodies against the interferon molecule [59]. This prompted researchers to develop albumin-free formulations [61] in which HSA is replaced by other excipients that can prevent oxidation, adsorption and aggregation. A new formulation of IFN-β1a [RNF or Rebif New Formulation, EMD Serono] was approved in Europe in August 2007 for the treatment of relapsing forms of multiple sclerosis. RNF is HSA-free and is produced by a CHO cell line in serum-free culture medium [62]. Overall results from a phase IIIb study suggest that RNF has improved local tolerability and immunogenicity compared with a historical study of the original formulation [63], and the authors attribute these benefits largely to the absence of serum components (HSA and FBS) from the preparation.

Optimizing the clinical outcomes following treatment with biopharmaceuticals is an issue of special relevance to immune-compromised patients struggling with chronic diseases. Repeated infusions over an extended period or over the lifetime of the patient increase the cumulative risk of pathogen transmission. Therapeutic monoclonal antibodies (mAb) have presented an innovative approach to the treatment of illnesses in which the immune system may be compromised, such as cancer, autoimmune diseases and inflammatory disorders [64]. Successive waves of technology have advanced mAb development from murine antibodies to chimeric, humanized antibodies and finally to fully-human antibodies. mAb manufacturing is trending towards the adaptation of cell lines to serum-free media, along with the removal of animal components from the purification and formulation processes [45, 65-67]. One of the first examples of this trend is the tumor necrosis factor alpha (TNFα) antagonist, which is approved for the treatment of several autoimmune diseases including rheumatoid arthritis and inflammatory bowel disease. The TNF antagonist has evolved from being a chimeric mAb produced from cells grown in media containg serum components (Remicade; Centocor) to a fully human anti-TNFα mAb that is plasma-free in both its manufacturing and formulation (Humira; Abbott). Since TNF plays a critical role in

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modulating the inflammatory immune response, patients treated with TNF antagonists are at special risk for serious infections, which further emphasizes the need for such therapies to be completely pathogen-free. In preventive therapies such as vaccines, minimizing the risk of pathogen transmission is also of paramount importance since vaccines are typically administered to large numbers of children and elderly individuals. Animal protein-free technology has been applied in the development of pandemic and seasonal influenza vaccines, in which embryonated egg-based technology is currently being replaced by mammalian cell lines grown in serum-free, protein-free medium. Examples include the Vero cell-based H5N1 pandemic mock-up vaccine that has completed preclinical development and is awaiting licensure (Baxter) and a new MDCK cell-based seasonal influenza vaccine that was recently licensed in Europe (Optaflu; Novartis).

In the aftermath of the vCJD outbreak in the UK, and with the recognition that prions can be transmitted through blood, access of potentially infected patients to dental and surgical care was impacted [68]. The UK government required the quarantine of equipment and procedure rooms used on patients who were at risk of having contracted vCJD from infected donors, including hemophilia patients who received plasma-derived factor concentrates. The prohibitive logistics and cost of dedicating equipment for at-risk patients may have made hospitals and dental providers less likely to offer standard services to these patients [68]. Problems such as these can be mitigated by providing previously untreated patients with products that have been manufactured without the addition of animal-derived components. Such products are not only an obvious choice for young, previously-untreated and unexposed patients, but should also be considered for patients with existing infections (such as HIV, HBV, or HCV) who must be concerned about the introduction of additional pathogens, which could further complicate a pre-existing infection [69].

Another important factor to consider is that the fear of exposure to potential pathogens in serum-containing therapeutics may cause some patients to forgo prescribed treatment. The availability of products with no risk of pathogen transmission may improve patient compliance, leading to better treatment outcomes and improved quality of life.

3 Response from regulatory agencies and physicians' organizations

Assuring the safety of recombinant therapeutic proteins is a primary concern of regulatory agencies, provider organizations and manufacturers. When alternatives to human- or animal-derived materials do not exist, the best measures available to assure product safety are to control the source of the raw material, test raw material for possible contamination by infectious agents, implement various virus-inactivation and removal steps, and test the end products to confirm absence of pathogens.

Following the BSE outbreak, strict requirements were imposed on manufacturers of biologics and medical devices regarding the country of origin for bovine-derived material used in manufacturing [http://www.fda.gov/cber/bse/bse.htm]. Drug companies were forced to find alternatives as the list of countries provisionally free of mad cow disease dwindled. The list of restricted countries was updated in 1998 to include not only those countries where BSE was known to exist, but also countries where no cases of BSE had been identified but which were considered by the US Department of Agriculture to have less restrictive import requirements than the US, and/or independent surveillance. For biologics manufacturers, CBER (Center for Biologics Evaluation and Research) now requires that manufacturers provide detailed information on the cell culture history, isolation, media, identity and pathogen testing of cell lines used in the production of biological products. Additional source control measures include expanded human donor screening and deferral guidelines. However, safety regulations set forth by governments vary between countries, and these variations are compounded by donor screening policies that may differ even within the same country.

As noted by the US Centers for Disease Control and Prevention (CDC), previous testing of source material was inadequate due to the routine failure of serologic screening to detect newly infected donors in the pre-seroconversion phase of infection. This remains the single greatest risk of transfusion-transmitted viral infections [24]. More sensitive tests have been instituted, such as PCR-based nucleic acid amplification testing (NAT), including minipool NAT and single donor testing (ID NAT). For HIV and HCV, NAT is expected to shorten the lag time, during which infection cannot be detected, from 22 to 12 days for HIV and from 70 to 14 days for HCV; but NAT is not able to completely eliminate the lag time [24]. Donated blood is routinely tested for 6 pathogens: HBV, HCV, HIV-1 and -2, HTLV-I and -II, Syphilis and WNV (when appli-

Table 2. Recommendations by regulatory agencies and physicians' organizations for the avoidance of human or animal proteins in drug manufacturing

Organization	Recommendation	Reference
The European Medicines Evaluation Agency (EMEA)	"When manufacturers have a choice the use of materials from [...] non-animal origin is preferred"	[74]
Committee for Proprietary Medicinal Products (CPMP- advisory to EMEA)	The European note of guidance: "when medicinal products prepared from human blood or plasma are administered, infectious diseases due to the transmission of infective agents cannot be totally excluded. This also applies to pathogens of unknown nature..."	[75]
The Medical and Scientific Advisory Council of the National Hemophilia Foundation (US)	Encouraging manufacturers to "avoid using human and animal proteins in manufacturing their products"	[76]
The UK Haemophilia Centre Doctors' Organisation	Guidelines to choose "recombinant concentrates [...] manufactured and formulated with the least addition of human or animal protein..."	[77]
The Association of Hemophilia Clinic Directors of Canada	Guidelines for "the elimination of animal and human proteins as stabilizers in the final formulation, or as nutrients in the cell culture, or in any other part of the process, should be considered as a goal in terms of the quality of products"	[78]

cable). NAT is used to detect HIV, HCV, and WNV. As new pathogens emerge, new tests are continually developed and implemented, such as the test for *Trypanosoma cruzi*, the agent of Chagas disease [70].

Methods for the inactivation and removal of viruses from blood products first evolved in response to the HIV epidemic in hemophilia A patients and became standard practice by the mid-1980s [71]. These methods include pasteurization, vapor heating, low pH, solvent/detergent treatment, separation/purification techniques (such as ion-exchange and immunoaffinity chromatography) and nanofiltration. These steps have helped to significantly reduce the frequency of infections originating from the blood supply, especially for those pathogens for which screening is also performed. However, the risk from novel or emerging pathogens must be taken seriously [72] and, as noted by the CDC, "in order to protect the safety of the blood supply and blood-derived therapies, it must be recognized that new pathogens will continue to emerge" [73]. UK health officials withdrew all blood and blood products obtained from donors in which vCJD was subsequently detected, and today, all plasma used in the UK to produce therapeutics is imported. The UK is among many countries (including Australia, New Zealand, Denmark, Ireland, Canada and Japan) that are switching to recombinant products as a standard for the treatment of hemophilia A, and regulatory agencies and physicians' organizations in North America and Europe have prompted drug and device manufacturers to find alternatives to human and animal raw materials and additives whenever possible (Table 2). The FDA and the International Conference on Harmonisation, which develops guidelines for the regulatory bodies of the European Union, Japan and the US, have provided documents guiding the sourcing, characterization and testing of raw materials derived from humans or animals, as well as guiding the evaluation of protein therapeutics for the presence of viruses [79-82]. The objective is to avoid transmission of adventitious agents, an issue that would not exist if animal-free substances were used in the manufacturing process. Because of regulatory concerns about serum and other animal-derived components, the same product can be manufactured in different formulations depending on the regulatory requirements of the country in which it is distributed. Aranesp (Amgen) is a recombinant human erythropoietin that has been modified to contain additional N-glycosylation sites to increase serum half life. Although Aranesp is available in both an HSA-containing and HSA-free formulation in the US, only the HSA-free formulation, containing polysorbate 80, is approved for use in Europe.

4 Discussion

Recombinant therapeutic proteins can be broadly categorized into blood factors, anticoagulants, growth factors, cytokines, hormones, vaccines, therapeutic enzymes and monoclonal antibodies. In each of these categories, evolution has occurred in the manufacturing and engineering of these products resulting in better therapeutic outcomes, such as enhanced safety, lower immunogenicity, increased half-life, improved bioavailability and alternative routes of administration. These enhance-

ments should have a positive impact on patient compliance and consequently on treatment outcomes.

The safety of biopharmaceuticals has come to the forefront for both patients and health care providers due to outbreaks of emerging pathogens, most notably HIV, HCV, vCJD, WNV and SARS, in multiple regions of the world [http://www.who.int/whr/2007/whr07_en.pdf]. The risk of pathogen transmission through the use of human- or animal-derived raw materials in the manufacture of pharmaceuticals was the major driver behind the development of PF technology. Blood-derived products were at a special risk of harboring infectious agents and thus present the most important example of the shift to safe alternatives produced by PF manufacturing technology. Patients with hemophilia were at particularly high risk for infection by blood-borne pathogens because they relied on plasma-derived or plasma-containing clotting factor therapies. This excessively high risk was first evidenced by the transmission of HIV-1 to approximately half the hemophilia population prior to the availability of a screening assay for that virus [83].

A number of measures have been implemented in recent years to ensure the safety of plasma derivatives. These measures include screening of blood donors, rigorous testing of plasma pools, incorporation of virus inactivation or removal steps into the manufacturing process and ultimately, the development of recombinant products [83]. These measures have greatly reduced the threat of pathogen transmission, but some risk remains because a number of recombinant products still incorporate plasma-derived additives during manufacture [19].

Although tests are available to screen for most known pathogens, no tests exist to screen the blood supply for infectious agents such as prions. Prions and parvovirus B19 may be markers for as yet undiscovered or unrecognized blood-borne infectious agents [MASAC#169]. The risk from unknown pathogens should not be neglected, as these agents may appear in the blood supply in the future and could have a significant impact on anyone receiving a therapy derived from blood or produced with blood-derived additives.

Most recombinant therapeutic proteins are injectables and many are used over long durations for the treatment of chronic diseases, in which case repeated infusions increase the cumulative risk to the patient. To completely eliminate the potential risk of transmission of any new or existing infections agent, recombinant therapeutic proteins should be produced in cell lines in the absence of human- or animal-derived proteins, processed with dedicated pathogen removal and/or inactivation steps, rigorously tested for lipid- and non-lipid-enveloped viruses, and packaged or stabilized in the absence of human- or animal-derived proteins.

The PF process often serves as a platform for the development of more advanced versions of existing drugs. Some of these drugs are genetically modified for greater efficacy, increased half-life, or lower immunogenicity, while others are tailored to be effective in specific groups of patients, or engineered to be effectively administered through less invasive routes. Erythropoeisis-stimulating agents, which were introduced in the late 1980s for the management of anemia in patients with chronic kidney disease [84], serve as a good illustration of the evolution of recombinant therapeutic protein manufacturing. First, a recombinant product replaced its blood-derived predecessors. In the next phase, a product with a longer half-life was developed (darbepoetin α or Aranesp). This product then evolved through conversion to an HSA-free formulation [85], followed by serum-free formulations of Epoetin α (outside the US, Eprex; Ortho Biotech) and Epoetin β (NeoRecormon; Roche). Most recently, a PF, PEGylated recombinant erythropoietin β with a longer half-life was developed and licensed in Europe (Mircera; Roche).

Advances in the optimization of serum-free media for the production of mAbs have succeeded in enhancing hybridoma productivity and product quality as compared to traditional cell culture media supplemented with FBS [45, 65-67]. Nonetheless, eliminating animal-derived components from media can significantly alter culture performance as well as post-translational protein modifications. The glycosylation pattern of an antibody molecule can affect its structural integrity, thus influencing both its biological function and its physicochemical properties. Heterogeneity in glycosylation patterns is evident in mAbs produced by hybridoma cells grown in media with different serum content [86]. In addition to influencing pharmacokinetics, glycosylation may influence both efficacy and safety, particularly immunogenicity [87].

Other factors that can influence the molecular integrity of a protein and affect its immunogenicity profile are physical degradation events such as unfolding, misfolding, fragmentation and aggregation [58, 88]. Biopharmaceuticals may become exposed to various conditions of stress, such as low pH or heat, which can potentially result in protein misfolding and the formation of amyloid-like structures [62]. The mechanisms by which different culture, formulation and storage conditions affect the physical stability of proteins are an active area of

research. Biopharmaceuticals require monitoring for potentially rare safety issues that might only be seen in studies of a large number of patients over an extended period of time. Thus, to ensure the safety of new PF products, phase IV post-licensure safety surveillance studies must be conducted to monitor products for efficacy as well as for rare, adverse events.

In reviewing the factors driving the development of innovative solutions to the use of human- and animal-derived products in the manufacture of biopharmaceuticals, the question remains whether regulatory agencies and industry in general are prepared to actively embrace changes such as adopting and/or mandating the use of a PF process for all new or reformulated therapeutic proteins. With the average cost of developing a biopharmaceutical product exceeding $1 billion [http://csdd.tufts.edu], manufacturers should make every effort to use the safest possible technologies, such as a PF manufacturing process. Given that no major infectious agent outbreaks have occurred in recent years, a false sense of security may be in place, but steps should be taken to avoid complacency. Such issues need to be addressed now that PF technology has matured and become more feasible, and this should be accomplished prior to the next major threat from a new, emerging pathogen.

Table 3. Selected list of approved recombinant therapeutic proteins produced in mammalian cell lines

Trade name	Recombinant product	Expression system	PF cell culture medium?[a)]	PF formulation?[a)]	Year of approval
Activase	Tissue plasminogen activator	CHO cells	No	Yes	1987 (US)
Epogen/Procrit	Erythropoietin (epoetin α)	CHO cells	No	No	1989/1990 (US)
Recombinate	Clotting factor VIII	CHO cells	No	No	1992 (US) 1993 (EU)
Kogenate; Helixate	Clotting factor VIII	BHK cells	No	No	1993 (US) 2000 (EU)
Kogenate FS; Helixate FS	Clotting factor VIII (sucrose formulation)	BHK cells	No	Yes	1993 (US) 2000 (EU)
Cerezyme	β-glucocerebrosidase	CHO cells	No	Yes	1994 (US) 1997 (EU)
Avonex	IFN-β-1a	CHO cells	No	Yes, in new formulation (EU 2003)	1996 (US) 1997 (EU)
Benefix	Clotting factor IX	CHO cells	Yes	Yes	1997 (US) 1997 (EU)
Rituxan (US)/ Mabthera (EU)	Anti-CD20 chimeric mAb	CHO cells	No	Yes	1997 (US) 1998 (EU)
Gonal-f	Follicle stimulating hormone (follitropin α)	CHO cells	Yes, in new formulation (EU) No (US)	Yes	1997 (US) 1995 (EU)
Simulect	Anti-IL2 receptor-α chimeric mAb	RFT5 (Murine hybridoma) cells	No	Yes	1998 (US) 1998 (EU)
Remicade	Anti-TNF α chimeric mAb	SP2/0 (Murine hybridoma) cells	No	Yes	1998 (US) 1999 (EU)
Herceptin	Anti-HER2 humanized mAb	CHO cells	Yes	Yes	1998 (US) 2000 (EU)
Enbrel	TNF α receptor – IgG fusion protein	CHO cells	No	Yes	1998(US) 2000 (EU)
Thyrogen	Thyrotropin α	CHO cells	No	Yes	1998(US) 2000 (EU)
Novoseven	Clotting factor VII a	BHK cells	No	Yes	1999(US) 1996 (EU)
Ovidrel or Ovitrelle	Human chorionic gonadotropin α	CHO cells	No	Yes	2000 (US) 2001 (EU)
ReFacto	B domain-deleted clotting factor VIII	CHO cells	No	Yes	2000 (US) 1999 (EU)
TNKase	Tissue plasminogen activator	CHO cells	No	Yes	2000 (US)
Aranesp	Darbepoetin α	CHO cells	No	No (US) Yes (EU)	2001 (US) 2001 (EU)

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Table 3. Continued

Trade name	Recombinant product	Expression system	PF cell culture medium?[a)]	PF formulation?[a)]	Year of approval
Xigris	Activated protein C (antithrombotic)	HEK293 (Human) cell line	No	No[b)]	2001 (US) 2002 (EU)
Osigraft (Osteogenic protein 1)	Osteogenic protein-1: bone morphogenetic protein-7	CHO cells	No	No	2001 (US) 2001 (EU)
Rebif	IFN-β-1a	CHO cells	No	No	2002(US) 1998 (EU)
Humira	Anti-TNF α human mAb	CHO cells	Yes	Yes	2002 (US) 2003 (EU)
Amevive	LFA-3-IgG fragment fusion protein	CHO cells	No	Yes	2003 (US)
Fabrazyme	α-galactosidase A	CHO cells	No	Yes	2003 (US) 2001 (EU)
Aldurazyme	Laronidase (α-L-iduronidase)	CHO cells	No	No	2003 (US) 2003 (EU)
Xolair	Anti-IgE humanized mAb	CHO cells	Yes	Yes	2003 (US) 2005 (EU)
Advate	Clotting factor VIII (plasma/albumin free)	CHO cells	Yes	Yes	2003 (US) 2004 (EU)
Raptiva	Anti-CD11a humanized mAb	CHO cells	No	Yes	2003 (US) 2004 (EU)
Avastin	Anti-VEGF humanized mAb	CHO cells	Yes	Yes	2004 (US) 2005 (EU)
Luveris	Luteinizing hormone	CHO cells	No	Yes	2004 (US) 2000 (EU)
Naglazyme	N-acetylgalactosamine-4-sulfatase	CHO cells	No	Yes	2005 (US) 2006 (EU)
Orencia	Ig-CTLA4 fusion (antirheumatic)	CHO cells	Yes	Yes	2005 (US) 2007 (EU)
Myozyme	Acid α-glucosidase	CHO cells	No	Yes	2006 (US) 2006 (EU)
Vectibix	Anti-EGFR human mAb	CHO cells	Yes	Yes	2006 (US) 2007 (EU)
Soliris	Anti-C5 (Complement protein) humanized mAb	NS0 (Murine myeloma) cells	No	Yes	2007 (US) 2007 (EU)
Mircera	Methoxy polyethylene glycol-epoetin β	CHO cells	Yes	Yes	2007 (US) 2007 (EU)
Rebif New Formulation	rh IFN-β-1a	CHO cells	Yes	Yes	2007 (EU)
Optaflu	Cell-based seasonal influenza vaccine	MDCK cells	Yes	Yes	2007 (EU)
Recothrom	Topical human thrombin	CHO cells	Yes	Yes	2008 (US)
Xyntha	B domain-deleted clotting factor VIII	CHO cells	Yes	Yes	2008 (US)

[a)] To the extent that information is disclosed by the manufacturer and available publicly on the FDA and EMEA websites.
[b)] Though final formulation is animal-free, bovine thrombin is used to activate the zymogen.

The authors acknowledge and thank Paul Woo, PhD and Sean Brugger, PhD for their thorough review of the manuscript.

The authors have declared no conflict of interest. The use of trade names is for product identification purposes only and does not imply endorsement.

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