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Review Article

Impact Factors in the Process Development for Therapeutic Antibodies

Xiaopei Cui, Xiangyang Zhu*

Department of Process Development, Huabo Biopharma, Shanghai, China

Email address:
Xiang.zhu@huabobio.com (Xiangyang Zhu), Xiaopei.cui@huabobio.com (Xiaopei Cui)

*Corresponding author

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Abstract: The research and development of therapeutic antibodies in the world is rapidly rising in recent years. It is a huge challenge to obtain a robust process and achieve the consistency of consistent product quality, because most of the antibody drugs are produced from mammalian cells of which the production processes are long and complex. To develop a robust and high yield production process for a biotherapeutic, one needs to have a general consideration of entire R&D project working flow. First of all, it is necessary to start with a good drug candidate, which not only meets the requirement for both the effectiveness and drug safety, but also suits for manufacture. Then, the expression system and host cell line used for the product expression need to be determined based on availability and regulatory requirements. Cell line development needs to combine with early upstream and downstream process developments as well as product quality assessment. Once a suitable stable cell line is selected, the Master Cell Bank (MCB) and the Working Cell Bank (WCB) are established according to the regulatory requirements, and tested for growth, production stability and cellular safety. Finally, process optimization and scale-up production are carried out to obtain a consistent production. Upstream process development is mainly to optimize a variety of process parameters, including physical parameters, chemical parameters, metabolite levels, and feed strategy and foam control; downstream process development focuses on product quality, recovery rate, and cost efficiency. For scale-up production, enterprises need to make decisions whether to use traditional stainless steel system or the single-use disposable system based on demands on product market potential and available technical supports. In this review, combined with the development of new technologies in recent years and the authors' personal working experience, the factors that need to be considered in process development are discussed.

Keywords: Therapeutic Antibody, Process Development, Quality Control

1. Introduction

Over the past three decades, therapeutic monoclonal antibodies (mAbs) have made astounding development. Sales of mAbs are expected to reach 125 billion US dollars by 2020 [1-2]. In 2017, of the top ten best-selling pharmaceutical products, six were monoclonal antibodies, of which Humira topped the list with $16 billion. In the past 10 years, the research and development of therapeutic monoclonal drugs in China has also rapidly risen. Some leading enterprises, such as Sansheng Guojian, Chengdu Kanghong, and Topallies and so on, have emerged. In the meantime, hundreds of other enterprises are rapidly following up [2-3].

It is a huge challenge to obtain a robust process and achieve the consistency of product quality, because most of the antibody drugs are produced from mammalian cells and the production processes are long and complex [4-5]. Therefore, how to develop a robust and high-yield production process that can be scaled up to meet the needs of the market is a key issue that every company must address. To develop a robust and consistent process, we need to have a general understanding of the entire R&D project flow: what the indications are; what the market may need; the route of administration (intravenous vs. subcutaneous); possible dosage and dosing frequency; operating space or time left by competitors, etc. From the above information, one can set up a

goal for designing a process development. In this review, combined with the development of new technologies in recent years and the personal working experience, the factors to be considered in process development are discussed.

2. Discussion

2.1. Candidate Drug Screening

Before proceeding with process development, it is necessary to have a general understanding of the drug candidates that will be developed. The first step to establish a robust process is to choose a good drug candidate.

At present, monoclonal antibodies (including Fc-fusion proteins) are the main biological products. Here we mainly discuss the process development of monoclonal antibodies. Currently in China, there are two main types of monoclonal antibody therapeutics under development: biosimilar and innovative. A biosimilar is a clinically and market proven drug similar to the reference product that has already been authorized for use. There is no issue of selecting drug candidate; however, companies should consider their ability to produce a highly similar product to match the reference product's quality, and the domestic market space and their own ability to obtain market shares. Therefore, the selection of a good drug candidate is mainly for a biological innovation therapeutic. To choose a better monoclonal antibody as a therapeutic, regardless of the technology used (including human monoclonal antibodies [6], murine monoclonal antibodies [7-8], rabbit-derived monoclonal antibodies [9], chicken-derived monoclonal antibodies [10], and monoclonal antibodies isolated using single-cell techniques [11]), it is usually a numbers game: the greater the number of candidate mAbs for the same target, the greater the range of options available.

For the drug candidate to enter into process development, it should meet some basic requirements; such as sufficient affinity (Kd) to bind to the target, highly specific target binding property (no off target binding), effective inhibition to block target signaling (IC50, IC80), and controllable operation freedom of intellectual property [12]. In addition, it should also meet the feasibility factors for process development and scale-up: such as high stability (Tm value), good solubility, low immunogenicity, favorable isoelectric point (ideally, pI = 5-6 or pI = 8-9) and no liabilities in Complementarity Determining Regions, CDRs (including N-sugar, methylation, and deamidation, etc.) [12-14]. The process development would become smoother only after fully considering the above comprehensive factors. The type of immunoglobulin (Ig) should be selected based on the indication area and whether the activities of ADCC (antibody-dependent cell-mediated cytotoxicity) and CDC (complement dependent cytotoxicity) are required for the mechanism of the treatment.

2.2. Establishment of Expression System and Stable Express Cell Lines

Once the drug candidate is selected and confirmed, the expression system and cell type need to be determined. There are many types of therapeutic biological product expression systems, such as *E. coli*, yeast, insect cells, and mammalian cells. Monoclonal antibodies are mainly expressed in mammalian cells that are close to human cells due to the complex post-translational modifications, especially glycosylation, which directly affects the antibody's efficacy, half-life *in vivo*, as well as immunogenicity and product safety. Commonly used cell lines are CHO, NSO, SPO and HEK293. According to market product analysis, CHO cells are the most widely used cell line. And CHO-K1 has been transformed into CHO-GS (Lonza, Sigma, etc), CHO-DG44C (Boehringer Ingelheim, Life technology), CHO-S (Life technology), CHO-M (Selexis), etc [15-16]. Judging from the maturity and stability of the expression system, most of the products are produced by the CHO-GS and the CHO-DG44 systems. In contrast, the period for obtaining a stable cell line by the CHO-S or CHO-M system is relative shorter (3 months), but the stability of cell lines for production remains to be confirmed by more mature products.

For any emerging biopharmaceutical firm, it's critical to choose a suitable expression system. Factors that influence the choice include: access to expression vectors and cell types, relevant requirements of the regulatory authorities, types of drug candidates (biosimilar or innovative), professional support and funding from corporate, objective requirements on product quality and output, and the speed for product development considering the competitive environment, etc [17-18]. Prior to development of the production stable cell line, profiling molecules produced from appropriate-scale transient transfection is highly valuable to gain understanding of the expression level and protein quality, and to anticipate the hurdles for upstream and downstream process development. In general, a monoclonal antibody with low transient expression has more difficulty obtaining a high expressing stable cell line [19-20]. Similarly, mAbs with poor quality (including aggregation, degradation, unexpected ratio of acid or base peaks, etc.) can also cause great difficulties in downstream process development. If serious problems are identified, protein engineering should be done as early as possible to optimize the drug candidate prior to initiating a stable cell line development.

At present, the technology for cell line development is rather mature. In particular, technological innovations such as the flow cytometer, cell imager, and Clonepix have made cell line development less tedious than the traditional screening process. And the expression level of biotherapeutics has also greatly improved, which plays a crucial role in reducing drug manufacturing cost. However, it should be pointed out that the overexpression of cell lines is often at the expense of a decline in product quality. Therefore, based on the idea of quality by design (QbD) [21], comprehensive quality and characterization analysis of the drug candidate should be performed in the early stage of cell line development, and suitable quality standards should be established as early as possible. For the process development of biosimilar drugs, it's of great importance to obtain satisfactory data of the

glycosylation patterns, acid-base peak ratio, molecular integrity, purification recovery, solubility, clarity, and impurities of the candidate drugs before the final cell line is determined. And multiple batches of the biosimilar products need to be compared with multiple lots of the reference drug to learn about the operational space of the biosimilar, and the similarity between the biosimilar and the reference product [22-23].

When selecting a cell line for development, the identity of the protein expressed from the cell line needs to be confirmed by various methods, especially by molecular weight studies through LC-MS/MS. Besides the product quality, the titer of the cell line in the current production state should be considered, and more importantly, the specific productivity or picogram per cell per day (PCD) should be monitored, which is the critical factor for an upstream process development to reach higher productivity. Once a suitable stable cell line is selected, the Master Cell Bank (MCB) and the Working Cell Bank (WCB) are established according to the requirements of regulation. The cell lines are tested for growth, production stability, and cellular safety. The stable growth and productivity must be maintained for more than 3 months and confirmed at genetic levels. Cellular safety assessments include detection of viruses and mycoplasma. In addition, MCB and WCB should be kept in different locations so that remediation can be done when an accident occurs.

2.3. Upstream Process Development and Scale-up

2.3.1. Upstream Process Development

The above-mentioned selection of a stable cell line not only depends on the titer of the cell line but also on the cell line productivity (PCD). When the PCD of a cell line is higher, the potential for increased production through process optimization is greater. Mammalian cells are produced in a variety of ways, such as adherent and suspension. Most systems would adopt suspension culture for the process development of monoclonal antibodies for the reason of the continuous innovation of technology and the convenience of scaled-up production. The optimization of the upstream process depends on three major factors: Expression system (expression vector and cell line) [24]; Optimization of process parameters; Selection of media and feeds and feeding strategy. Therefore, obtaining a stable cell line is only one-third of the process development work. Upstream process development is mainly to optimize a variety of process parameters [25]: physical parameters including temperature, gas flow, agitation speed; chemical parameters including dissolved oxygen and carbon dioxide, pH, osmotic pressure, redox potential; metabolite levels including basal media, amino acids and waste (by-products), as well as feed strategy and foam control. Changes in these factors can affect protein yield and product quality, particularly post-transcriptional modifications which may impact the product efficacy and safety. The distribution of glycosylation of a monoclonal antibody can be adjusted by various components in the culture medium. For example, galactose would increase the proportion of glycosylation, while N-acetyl-D-glucosamine (GlcNAc) is the opposite. Temperature shifts and the addition of non-essential amino acids have effects on glycol structure, especially the modification of fucose, galactose, and sialic acids, and temperature downshifts could suppress the levels of acidic charge variants [26].

There are many parameters that affect the expression level of antibodies. For example, high viable cell density (VCD) increases antibody production, but unrestrained cell division hyperplasia may play an opposite role. The culture pH and the fermentation time of these process parameters are the most critical parameters to determine protein purity. The Aggregations content is a critical attribute for therapeutic monoclonal antibodies, because the aggregations could increase the immunogenicity of the antibody, reduce its efficacy and half-life in vivo, and may even affect the safety of the product. Aggregations are generally formed in the endoplasmic reticulum by the asymmetric pairing of heavy chain and light chain [27]. In general, higher expression of light chains reduces the formation of such polymers.

Traditional process development is carried out by a large number of shake flasks or 1 to 3L fermenters. In recent years, the emergence of high-throughput micro-bioreactors (such as the Ambr system) has increased the throughput of process development and reduced fermentation volume and the labor. The Ambr system is an automated micro-bioreactor system that simulates classic lab-scale bioreactor and could enable automated process development. In comparison with traditional laboratory bioreactors, Ambr systems can provide efficient, consistent and scalable cell culture processes. After the process optimization is relatively stable and preferred parameters are obtained, more than 3 batches of process consolidation are required, which can be done in 2-50L volumetric fermenters. On this basis, with repeated batches of scale-up tests, the process parameters are determined and the process can be locked for a scale up production. For a process development technician, it is important that the process is robust and repeatable. Once the process is locked, the production department cannot change the process parameters at will.

Continuous process development for later production stages (such as Phase III clinical trial and commercial production) is encouraged under good design of quality and is normally performed by the process development department rather than the production department. When a major process change occurs in the later stage, one should seek for the regulatory approval, and proper comparability studies are required to ensure the product quality unchanged.

There is much debate about the selection of culture medium [28-29]. At present, chemically defined media are mostly used for the production of monoclonal antibodies. The main components of chemically defined medium include amino acids, vitamins, trace elements or inorganic salts, and insulin or insulin-like growth factors. The optimization of cell culture media is usually based on the specific cellular metabolism and nutrient consumption. Not all production cell lines can be highly expressed in chemically defined media, and sometimes animal-free hydro lysates added to chemically defined media

would effectively increase cell density, thereby increasing production efficiency. One of the most commonly used feed development methods is to concentrate the basal medium. One needs to pay attention to the consumption of nutrient components, the density of the cell growth, viability, and the accumulation and balance of by-products when optimize the feed composition and feed strategy. For example, lactic acid and ammonia are common by-products, and these two by-products could be reduced by keeping low concentration of the glucose and glutamine and by frequently or continuously replenishing the excipients. However, due to high cost and inconvenient operational, continuous feed strategies are not well suited for large-scale production. The most widely used method is to add the feed solution to the bioreactor according to a fixed time and a limited parameter change, so that the operation is simple and easy to expand. It is necessary to point out that the development of medium and feed should fully consider whether there are residues of harmful substances and virus contamination contacting with the expression components, whether the medium and feed ingredients meet the requirements of GMP, as well as its cost and the stability of the supply chain.

2.3.2. Scale-up of Upstream Process

After the upstream process is mature and stable, the process needs to be scaled up. A scale-up process should be designed on the premise of reducing the impact on product quality. The impact of the expansion of production scale should be taken into consideration during the process development, and process steps that are not suitable for scale up should be avoided or the equipment should be updated if necessary. Before scale up, one would need to decide which type and what scales of fermentation tanks are used; the traditional stainless steel system or the single-use disposable system? Both systems have advantages and disadvantages, and there is not a standard answer [30]. Enterprises should make decisions based on their own research projects, business plans and scale, and the availability of funds, market potential. In our opinion, the single-use system is more suitable for emerging biopharmaceutical companies. In the past 10 years, single-use systems have been developed rapidly due to their advantages such as simplicity, flexibility, speed, and low initial setup cost. It has been favored by more and more companies [31]. In the application of single-use system, special attention should be paid to leachable and extractable detection [32], because of the contact surface of plastic equipment is significantly larger than the traditional stainless steel process [28]. At present, the scale of single-use system has reached 2000 L, which would meet the market supply of most biotherapeutics. The main reasons are as follows: (1) With the increasing improvement of biotechnology, the new generation of biological drugs often have higher binding affinity, better efficacy and longer half-life, resulting in a decrease in the dosage of clinical utilization; (2) With the development of personalized and precision medicine, and the perfection of clinical biomarkers, the drug delivery is with a higher accuracy, resulting in the narrowing scope of delivery populations of specific medicine and a significant reduction in market demand for a single drug; (3) For the fierce market competition, the emergence of any new target would promptly have a large number of competitors, leading to market share redistribution.

The scale-up production process includes seed resuscitation, seed expansion, and fermentation production. Each step has its key process parameters, which will determine the speed of cell growth, cell viability, product yield, and product quality. Therefore, each step of the amplification process needs to ensure the consistency of critical process parameters, so that the scaled-up production can achieve the desired goal. The bioreactor operating parameters can be divided into [33]: volume-related parameters such as working volume, feed volume, agitation, aeration, and volume-independent parameters such as pH, dissolved oxygen, temperature. The general strategy of amplification is to scale up the parameters related to the volumetric quantity while keeping the volume-independent parameters as the small-scale processes. Process amplification should pay attention to whether the scale-up involves the change of process. If there is any change, the consequences of such changes should be evaluated. In addition, changes in the production site should better be avoided.

2.4. Downstream Process Development and Quality Control

The traditional antibody downstream process has been well developed and is relatively mature. Typically, cell removal (such as deep filtration) is performed in the beginning; the clarified supernatant is then further purified by protein A resin affinity chromatography, which is followed by low pH (~3.5, 1-2 h) viral inactivation [34-35]., and after filtration, two polish purification steps (ion exchange resin and hydrophobic exchange resin) are done to remove process related impurities, such as residual DNA, endotoxin and other host cell proteins, and product related impurities, such as polymers and degradation [36]. Finally, the intermediate product is concentrated by ultrafiltration to a stock solution that meets the formulation concentration. The acceptance criteria of downstream process optimization are mainly the consistency of product quality (monomer/polymer, degradation products, impurities, glycosylation and charge heterogeneity, biological activity, etc.), and the recovery rate [37-39]. Therefore, to establish a stable downstream process for a specific monoclonal antibody drug candidate, it is necessary to start the process optimization as early as possible, and it is better to start before the determination of the cell line selection. If conditions permit, a small-scale, high-throughput downstream purification technology platform could be established, so that one can use the comprehensive data of the upstream and downstream process results to select the final cell line.

In recent years, biotherapeutics for both bi-specific antibodies [40-41] and antibody-drug-conjugation (ADC) products have been rapidly developed [42]. Compared with conventional monoclonal antibodies, these molecular structures are much more complex, and it is rather difficult for the upstream process to obtain highly uniform monomer products, which are often accompanied by higher levels of degradation

products and/or multimer products. It brings great challenges to downstream processes as well. A decent downstream process should be able to effectively remove these impurities, maintain a high recovery rate, and meet the clinical and marketing supplies. Many major biopharmaceutical companies in the world are working hard to solve those issues to develop bispecific antibody or ADC biotherapeutics. Taking Roche's well-known Cross-Mab molecule as an example, a great deal of manpower and material resources were spent to develop a relatively stable cell line, upstream and downstream processes, and finally complete their Phase I clinical trial [41]. It should be pointed out that early downstream process development needs to consider its continuation from each step to meet the requirements for the GMP production. In the research and development stage, there are many methods and tools for removing impurities, and there are many resins of different qualities to choose from. However, many times, a process solves the problem of impurities in an early laboratory, but it could not be industrialized because the resin used does not meet the GMP production standards [43-46]. If conditions permit, the life cycle of a resin should also be verified as soon as possible.

Antibody drugs are mainly administered by intramuscular injection and intravenous injection. According to the stability of the antibodies, the general formulations are mainly aqueous; some individual antibody drugs that are unstable in aqueous solution can be first made into freeze-dried powder, and then dissolved in aqueous solution before clinical use. In general, antibody drug used for oncological diseases are administrated by an intravenous injection, which is relatively easier for formulation development, since it often does not require high concentration. However, antibody drugs used for chronic diseases such as Rheumatoid Arthritis or Psoriasis are often administrated through intramuscular injection, which requires a higher concentration to limit the volume of the injection. Therefore, it is rather challenging to develop a highly concentrated solution drug, which is not only dependent on the critical quality attributes of the particular drug candidate, but also requires comprehensive formulation process development. Formulation development is a very complicated process, which is not discussed in this article. It should also be noted that all buffers and excipients in the formulation ingredients must comply with pharmacopoeia standards and requirements.

2.5. Coordination and Cooperation Among Various Departments in Process Development

Process development is not only the development of upstream and downstream processes, but also the overall coordination of multiple functional departments such as quality analysis, formulation development and filling. Table 1 is a summary of work experience. The table shows that each functional department should participate in the process development as early as possible. For example, downstream purification starts immediately after transient expression so that the screening of cell lines can be guided based on the quality data of the product and the result of the purification. Similarly, quality control (QC) analysis should assess critical quality attributes (CQAs) as early as possible and establish and provide with a stable analysis platform. QC analysis can not only provide with early analysis of glycosylation, acid-base peaks, and purity, but also provide with help in the selection of high-expressing and high-quality cell lines. The quality data for each step also ensure each step moves forward in the downstream process development, which helps in the selection of purified resins and chromatography parameters. On the other hand, early development of formulations not only saves time, but also provides the potential buffer condition for the drug substance in the downstream process [14]. It must also be pointed out that quality assurance (QA) work covers the entire process development, including equipment validation, process validation, cleaning validation, quality process determination, raw material testing and balance, batch record release, and standard operating procedure (SOP) management.

Table 1. *Coordination and cooperation among various departments in process development.*

Process Stage Department	Expression Vector	Cell Pool Establishment	Stable Cell Line Establishment	Process Development	Toxicology and IND Generation	Filling
Upstream	Plasmid/cell transient	Pressure screening	Screening of single cell, amplification, stability	Development of fermentation conditions, medium selection	GMP fermentation of 3 batches	
Downstream	Purification of transient protein	Preliminary purification, process development	Determination of basic purification process, Selection of various resins	Downstream process optimization, amplification and process locking	GMP purification of 3 batches	
Formulation		Early formulation development (buffers and excipient screening, packaging materials research)		Determination of pH and concentration, stability research	Stability studies, determination of final formulation	Formulation filling
Quality Analysis		Early analysis methods to determine glycoforms, acid and alkali peaks, deoxygenation, deamination, purity, isoelectric point		Solubility, viscosity, repeatability between batches	Stability study on stock solution of 3 batches	Stability study on formulation of three batches

3. Conclusion

In summary, the establishment of a mature biotherapeutic production process is very complex and requires the cooperative development of multi-functional departments. There are many influencing factors. First, there must be a high-expressing cell line; continuous and stable high-yield

upstream and downstream processes; high-quality products that meet the regulatory (CDA, FDA, EMA) requirements; and effective analytical and fit-for-use methods. Second, the developed process should be easy to scale up and can stably support market production for 10 to 20 years, and the formulation should be suitable for injection and has long stability to ensure its shelf-life. Finally, one needs to have an overall consideration about the production speed and cycle, annual production capacity and production costs, and whether all the necessary raw materials and consumables have stable long-term protection. Only when all the above aspects are optimized, it becomes a mature process.

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