Cell Culture Technology for Pharmaceutical and Cellular Therapies

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Outline

1. Introduction to Monoclonal Antibodies
2. Production of Monoclonal Antibodies
3. Introduction of Centocor
4. Autoimmune disorders
5. Development of Monoclonal Antibody based Pharmaceutical Therapies
Immune Response as First Line of Defense

When a pathogen (bacteria, foreign proteins, virus,..) enters the blood stream it is recognized, attacked, and eliminated by a sophisticated defense mechanism: Body's Immune Response

Immune System: The Body's First Line of Defense

Lymphatic vessels form a circulatory system that operates in close partnership with blood circulation.

Organs and tissues of the immune system dot the body in a protective network of barriers to infection.
The antibody – a.k.a “Immunoglobulin”

Antibodies are produced by B-cells as part of immune response. Each antibody is specific to a specific antigen.

- The Variable region is different for each antibody and determines its specificity.
- The Constant region is identical for each type of antibody and allows recognition by your immune cells.

Evolution of Antibody Technology: Development of Antibody Based Medicines

- Utilization of antibodies as medicines took some time:
  - B-cells cannot be expanded in vitro for practical purposes
  - It is difficult to find out which B-cell makes a specific antibody
- Kohler and Milstein discovered Hybridoma Technology and cloning in 1975: A revolution in antibody technology
  - Hybridoma cells (a fusion of B-cell and myeloma cell) can be expanded indefinitely
  - Utilization of cloning techniques allows to isolate cells that make a specific antibody: These antibodies are called Monoclonal Antibodies (Mab)
- Expansion of hybridoma cells in vivo (mouse) or in vitro (bioreactors) allowed the development of first MAbs
- Over the last 30 years antibody technology was further developed
  - Engineering of antibodies to make them more “human”
  - To use cell lines other than hybridoma cells (CHO, NS0, etc)
  - The use of antibody fragments and fusion proteins
  - The use of antibodies for targeted drug delivery
**Hybridoma Technology**

- Antigen
- Cells fuse to make hybridomas
- Hybridoma cells grow in culture
- Individual hybridoma cells are digested
- Desired clones are cultured and frozen
- Monoclonal antibodies are purified
- Antibody-producing plasma cells
- Cancerous plasma cells

**Engineering of Antibodies**

- Murine IgG
- Chimeric IgG
- Humanized IgG
- Fully Human IgG

“Humanization” of antibody minimizes/eliminates immune reaction when injected to the patients

Current Products in Development
Monoclonal Antibodies as Medicines

- There are 18 approved antibody treatments in the market for:
  - Autoimmune disorders
  - Cancer
  - Asthma
  - Organ rejection

- Sales of antibodies is expected to be $13 Billion in 2005
- There are 500 new antibody products in development
- There are 75 new antibodies in clinical trials
- Sales of antibodies is expected to be $26 Billion in 2010
- Some of the indications require as high as 2,000 kg/year product
- These antibodies are produced in large (20,000L) bioreactors

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Therapeutic Antibodies Approved to Date

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See the list on the website: [Therapeutic Antibodies Approved to Date](http://www.nature.com/nbt/2005/23/9/abs/nbt0905.html)

Sadettin S Ozturk

Production of Monoclonal Antibodies

![Diagram of monoclonal antibody production process](image)

Monoclonal Antibody Production Process
Bioreactor and Product Capture

- Preculture
- Inoculum Bioreactor
- Production Bioreactor
- Clarification
- Frozen eluate
- 0.2 μm
- Concentration
- DPC Protein A sepharose
- Harvest
Production Bioreactors: Continuous Perfusion Operation

- Cells are retained in the bioreactor by physical means
- Cells grow, stay in the bioreactor, and produce proteins
- Media is added and harvest is collected continuously
- Can be operated for months
- Usually compact bioreactors (1000L)

Production Bioreactors: Batch Operation

- Inoculate with media and cells
- Cells grow and produce proteins
- All of the contents are harvested after typically 2 weeks
- Usually very large bioreactors (20,000L)
Purification of Monoclonal Antibodies using Column Chromatography

Concentration and Diafiltration of Monoclonal Antibodies
Centocor: An Antibody Company

25 years of innovation
Our Founding. Our Future.

1979 Centocor Founded
1991 CENTOXIN Launched
1995 ReoPro® Launched
1998 REMICADE® Launched
1999 Merger with Johnson & Johnson
2001 $1 billion Sales
In the past five years, sales have grown from $500 million to over $3 billion.

**Centocor Products**

- **Launched 1998**
  - Approved in over 80 countries World Wide
  - 2004 WW sales $2.63 Billion
  - Indications: RA / CD / AS / PsA/UC

- **Launched 1995**
  - Approved in over 50 countries World Wide
  - 2004 WW sales $363 Million
  - Indications: PCI
REMICADE® Approvals


Rheumatoid Arthritis – signs and symptoms
Crohn’s Disease

Rheumatoid Arthritis – structural damage
Psoriatic Arthritis

Rheumatoid Arthritis – physical function in failed methotrexate patients
Ankylosing Spondylitis

Rheumatoid Arthritis – structural damage
Psoriasis (OUS)

Crohn’s Disease – luminal CD

Crohn’s Disease – fistulizing

Psoriatic Arthritis

Rheumatoid Arthritis – signs & symptoms of RA, inhibiting x-ray diseases progression, and improving physical functioning in patients not previously treated with methotrexate

Number of Patients Treated Worldwide With REMICADE®
Autoimmune disorders: What Are They?

Disorders caused by an immune response against the body’s own tissues. Immune system disorders occur when the immune response is inappropriate, excessive, or lacking.

- Rheumatoid arthritis
- Crohn’s Disease
- Psoriasis
- Multiple sclerosis (MS)
- Systemic lupus erythematosus

Autoimmune disorders: Rheumatoid Arthritis

Inflammation begins in the tissue lining your joints and then spreads to the whole joint (hand joints are the most common site, but it can affect most joints in the body)

- muscle pain
- deformed joints
- Weakness
- Fatigue
- loss of appetite
- weight loss
- becoming confined to bed in severe cases
Autoimmune disorders: Crohn's Disease

- Chronic autoimmune disease where immune cells attack any part of the gastrointestinal tract
- The lining of the intestine may ulcerate and form channels of infection, called fistulas
- *Ulcerative colitis* is a similar inflammation of the colon, or large intestine

Autoimmune disorders: Psoriasis

- Immune-mediated, genetic disease manifesting in the skin and/or the joints
- Psoriasis and psoriatic arthritis affect more than 4.5 million people in the United States
- A person’s quality of life—including emotional health—can be seriously jeopardized
Autoimmune disorders:

Multiple sclerosis (MS)
- weakness and trouble with coordination, balance, speaking, and walking
- paralysis
- Tremors
- numbness and tingling feeling in arms, legs, hands, and feet

Lupus
- swelling and damage to the joints, skin, kidneys, heart, lungs, blood vessels, and brain
- “butterfly” rash across the nose and cheeks
- rashes on other parts of the body
- painful and swollen joints
- sensitivity to the sun

Autoimmune disorders:

A lot of things can go wrong in the immune system to result in autoimmune disorders
- T cell proliferation and interferon production
- Differentiation of T-cells
- Cytokine production
- Cytokine, receptor binding
- B-cell differentiation
- Antibody production
- Migration of cells to the tissue

Antibodies can be used to intercept or block these events
The use of antibody based treatment for Psoriasis

Psoriasis activity before and after treatment with a specific antibody: 0.1 mg/kg dose (1 week post-treatment [baseline not available] and 16 weeks post-treatment); 1.0 mg/kg dose (baseline and 16 weeks post-treatment)


Development of Antibodies for Pharmaceutical Therapies
Antibody Development and Commercialization Process

Clinical Development
- Early Development
  - Target Research
  - Preclinical Studies
  - Phase I / II Clinical Trials
- Late Development
  - Phase III Clinical Trials
  - Process Validation
  - Review Approval
- Submit BLA

Drug Development
- Cell Line Selection and Purif./Form. Dev.
- Clinical Manufacturing
- Process Validation
- Launch Preparation

Steps in Drug Development

Discovery Research
- Identify Molecular Target
- Create New Molecular Entity

Clinical
- Initiate Clinical Trials
- Make Clinical Supplies
- Develop Formulation

Pharmaceutical Development
- Cell Line Development
- Media Development
- Bioreactor Process Development
- Develop Purification Process
Cell Line Development

1. Clone product gene cDNA
2. Develop expression vector
3. Insert into expression plasmids
4. Transfection
5. Host Cell
6. Evaluate in Bioreactors
7. Development Cell bank (DCB)
8. Master Cell bank (MCB)
9. Master working Cell bank (MWCB)
10. Amplify, Clone, Select
11. Clone selection for high producing cells

Media Development

- Cell culture medium contains:
  - Salts, trace elements, glucose, amino acids, other nutrients, vitamins, buffers, etc.
- Early media formulations used serum or other animal derived proteins (albumin)
- Issues related to safety (BSE), availability, and cost became driving force to eliminate serum and to develop animal product free (APF) (safer and economical)
- Today chemically defined medium (CDM) is a reality for many cell culture based processes (consistent and traceable)
- Most of the companies use specially formulated in-house proprietary media formulations for their processes (independence)
Bioreactor Process Development

• Advances in biochemical engineering made it possible to grow animal cells in conventional bioreactors (no need for specialized systems)
• Today stirred-tank based bioreactors are in operation at sizes up to 20,000L
• Batch, fed-batch, and perfusion process options are in use for commercial production
• It is possible to get 5 g/L titers in fed-batch and about 50 MM cells/mL in perfusion
• Bioreactor process development involves
  – Optimization of culture environment (pH, temperature, DO, CO2)
  – Optimization of media exchange rates
  – Development of feeding solutions and feeding strategies

Process Development Options
Case Study: Development of a Fed-batch Process for Monoclonal Antibody Production

- Cell line: CHOK1SV
- Glutamine Synthetase selection system
- Animal product free medium
- Fed-batch process in stirred tank bioreactors
- Optimized pH, temperature
- Optimized feeding schedule
- Process Scale-up
- Consistency

Cell Line Development: Introduction/Selection System

Glutamine Synthetase (GS) catalyzes the biosynthesis of glutamine from glutamate and ammonia, providing the only pathway for L-glutamine formation in the cell

\[
\text{Glutamate} + \text{NH}_3 \rightarrow \text{L-glutamine}
\]

MSX

MSX = L-Methionine Sulfoximine

In the absence of glutamine, the GS enzyme is essential for cell survival.
**Cell Line Development: Schematic Overview of Plasmid Construction**

- Start with a research cell line (expressing < 20 mg/L)
- Isolate RNA, reverse transcribe to generate cDNA
- Use sequence information from genomic constructs to design PCR primers to isolate specific HC and LC cDNAs
- Clone cDNAs into Lonza GS vectors, pEE 6.4 and pEE 12.4
- Construct a GS ‘double-gene’ plasmid

**Cell Line Development: Process of Developing High Producing GS-CHO Cell Lines**

- **STATIC CULTURE**
  - Transfections
    - 3-6 weeks
    - 200-300 transfectants
    - 96w plates
    - Rank order clones by 96w single-point ELISA
    - 24w plates
    - 60-100 transfectants
    - Rank order clones by 24w Neph. (overgrow)
- **SUSPENSION**
  - 8 weeks
  - 3-10 parental cell lines
  - Adapt to CD-CHO
  - 3-10 parental cell lines
  - Perform shake flask growth profiles
  - 6 weeks
  - 1-3 parent cell lines
  - Prepare and test DCBs
  - 12-16 weeks
  - 1-3 parent cell lines
  - Bioreactor process development
- **SUBCLONES**
  - 3 weeks
  - 30-100 subclone cell lines
  - Subclone
  - 3 weeks
  - Rank 24w (Neph.)
  - 3-10 subclone cell lines
  - Adapt to CD-CHO
  - 3-10 subclone cell lines
  - Perform shake flask growth profiles

- **~ 4.5 months**
- **~ 6 months**
**Cell Line Development: Clone Selection**

Immuno-precipitation method for rapid selection of high expression/secretion clones:


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**Automated Halo-Colony Picking**

- ClonePix interior
- HEPA filtration
- CCD camera
- 1µm encoders
- Wash and sterilise
- Stacker for microplates
- Holder for 5 Culture dishes
Cell Line Development: Transfection and Colony Screening

Rank Order Clones: 96w ELISA / 24w Nephelometry

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<th>Clone #</th>
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<th>24w Neph Titer (mg/L)</th>
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Specific Productivity

\[ y = 18.055x \]
\[ R^2 = 0.9474 \]

\[ y = 17.049x \]
\[ R^2 = 0.9445 \]

Cell Line Development: RESULTS

- Expand in 24w, T-flasks, and cryopreserve
- Adapt highest expressing cell lines to APF medium (CD-CHO)
- Adapt to suspension culture in shake flasks
- Perform 10-passage stability study
- Perform growth profiles in shake flasks in APF medium
- Transfer cell line(s) to bioreactor process development group
Fed-batch Process Development: Temperature and pH Optimization in Batch Cultures

Fed-batch Process Development: Temperature and pH Optimization in Batch Cultures
Fed-batch Process Development: Temperature and pH Optimization in Batch Cultures

Fed-Batch Process Development: Feeding Strategies

- GS-CHO Cell line producing a fully human antibody
- Animal Product Free Medium
- pH, DO, and temperature set-points from batch optimization study
- Feeding solutions include
  - Glucose, plant hydrolysate, MEM, NEAM, Vitamins, Specially formulated cocktails
- Feeding strategies include daily additions of pre-determined amounts to the bioreactor
Fed-Batch Process Development: Feeding Strategies
CD CHO Fed Batch Viable Cell Density Comparison

Glu, BRX, Nucleosides, PHyd

Glu, PrHyd, MEM, NEAM, GS

BRx=MEM+NEM+Vitamins
PHyd=Plant Hydrosylate

Fed-Batch Process Development: Feeding Strategies
CD CHO Fed Batch Antibody Comparison

Glu, BRX, Nucleosides, PHyd

Glu, PrHyd, MEM

BRx=MEM+NEM+Vitamins
PHyd=Plant Hydrosylate
In-process Testing: Agilent 2100 Bioanalyzer

Non-reduced

Reduced

Process Scale-up and Commercialization
Process Consistency: SDS-PAGE

Reduced

Non-Reduced

Process Consistency: IEF
Conclusions

1. Monoclonal Antibodies evolved over the years to become an essential part of biotechnology
2. Monoclonal Antibodies can be used as an effective therapy for immune disorders
3. There are several processing options for the manufacture of Monoclonal Antibodies. The final choice may depend on a variety of reasons
4. Development and manufacturing of Monoclonal Antibodies require extensive optimization, consistency, and comparability studies
5. It can be tedious, frustrating, costly, and very risky, but making a drug that can help people’s life is worth it.